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**Le décryptage omique de l'hétérogénéité de
l'adénocarcinome pancréatique :
de la pailleasse au lit du patient.**

Omics deciphering of pancreatic ductal adenocarcinoma heterogeneity :
from bench to bedside.

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SOMMAIRE

Introduction	5
1. PANCREAS : PHYSIOLOGIE ET ANATOMIE	5
2. LE CANCER DU PANCREAS	5
3. LA PREVENTION	13
4. LES POPULATIONS A RISQUE	17
5. HETEROGENEITE BIOLOGIQUE	21
6. LE PROJET PaCaOmics (« Pancreatic Cancer et techniques de Omics »)	31
 Premier axe de recherche : l'étude multi-omique au service du décryptage de l'hétérogénéité de l'ADCP	 41
 Deuxième axe de recherche : comment améliorer la prise en charge thérapeutique des patients par le biais des résultats de laboratoire : <i>from bench to bedside</i>	 51
 Discussion	 57
 Perspectives	 61

Introduction

1. PANCREAS : PHYSIOLOGIE ET ANATOMIE

Le pancréas est constitué de cellules endocrines, réunies en îlots de Langerhans, et de cellules exocrines. Les fonctions du pancréas sont donc doubles : endocrine, pour la régulation glycémique par la sécrétion d'insuline et de glucagon ; et exocrine, par la sécrétion d'enzymes de la digestion (lipase, amylase), et par la régulation hydro-électrolytique (bicarbonates). Son origine embryologique est mixte (**Figure 1**) : un bourgeon ventral donnera le crochet pancréatique et l'ampoule hépato-pancréatique se jetant dans le duodénum, et un bourgeon dorsal sera à l'origine d'une partie de la tête, du corps et de la queue du pancréas. Cela a un intérêt dans les pathologies ainsi que l'approche chirurgicale du bloc duodéno-pancréatique. Sa position anatomique (**Figure 2**) est particulière puisqu'il s'agit d'un organe profond de l'abdomen, rétropéritonisé, plaqué sur le billot rachidien, à proximité des plexus nerveux coéliquaux, qui seront donc facilement envahis par une tumeur, donnant lieu à des douleurs dorsales de type « solaire ».

2. LE CANCER DU PANCREAS

2.1. Terminologie

Il existe différentes histologies de tumeur malignes du pancréas, selon le type cellulaire d'origine de la tumeur : les cellules acinaires donneront des adénocarcinomes à cellules acineuses (1-2% des tumeurs exocrines), les cellules du tissu endocrine formeront quant à elles les tumeurs neuro-endocrines

Les cellules canalaire, responsables de la sécrétion de mucus pour la fonction exocrine, formeront des adénocarcinomes canalaire, les plus représentés (85% des tumeurs exocrines), et fréquemment regroupés sous le terme générique de « cancer du pancréas ».

Figure 1 : embryologie pancréatique : ébauche ventrale et dorsale, rapports avec l'arbre biliaire et le duodénum.

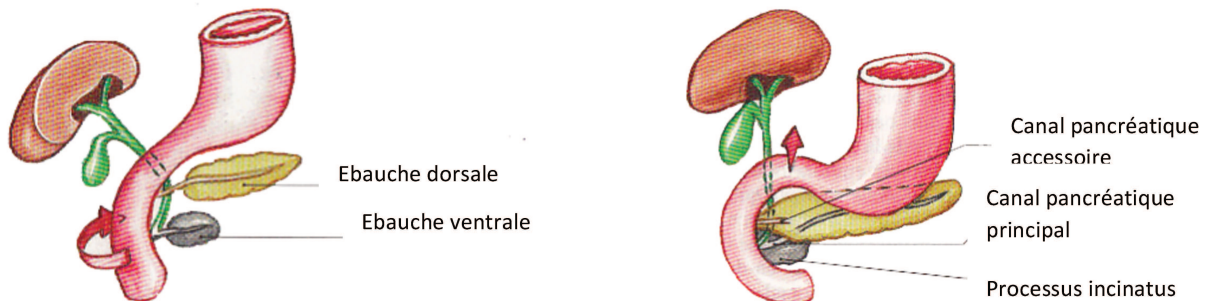
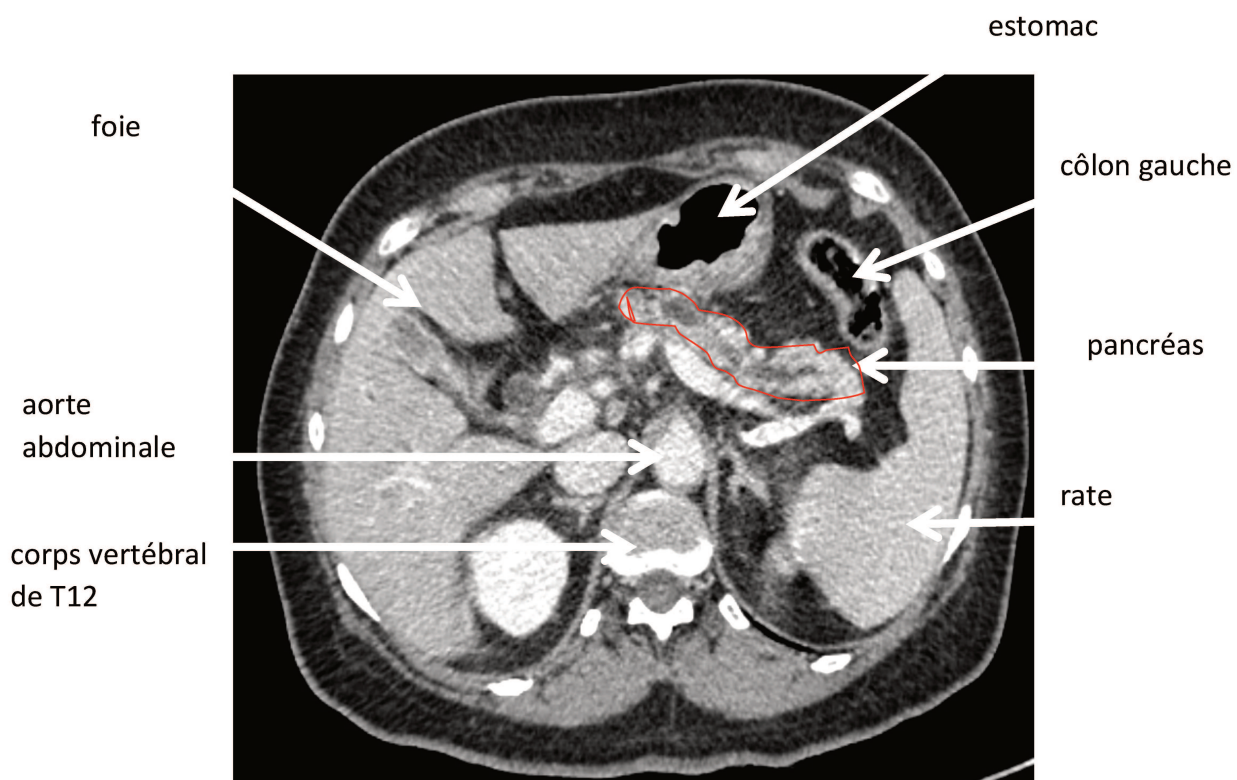


Figure 2 : position postérieure du pancréas visible sur une coupe scannographique transversale



2.2. Epidémiologie

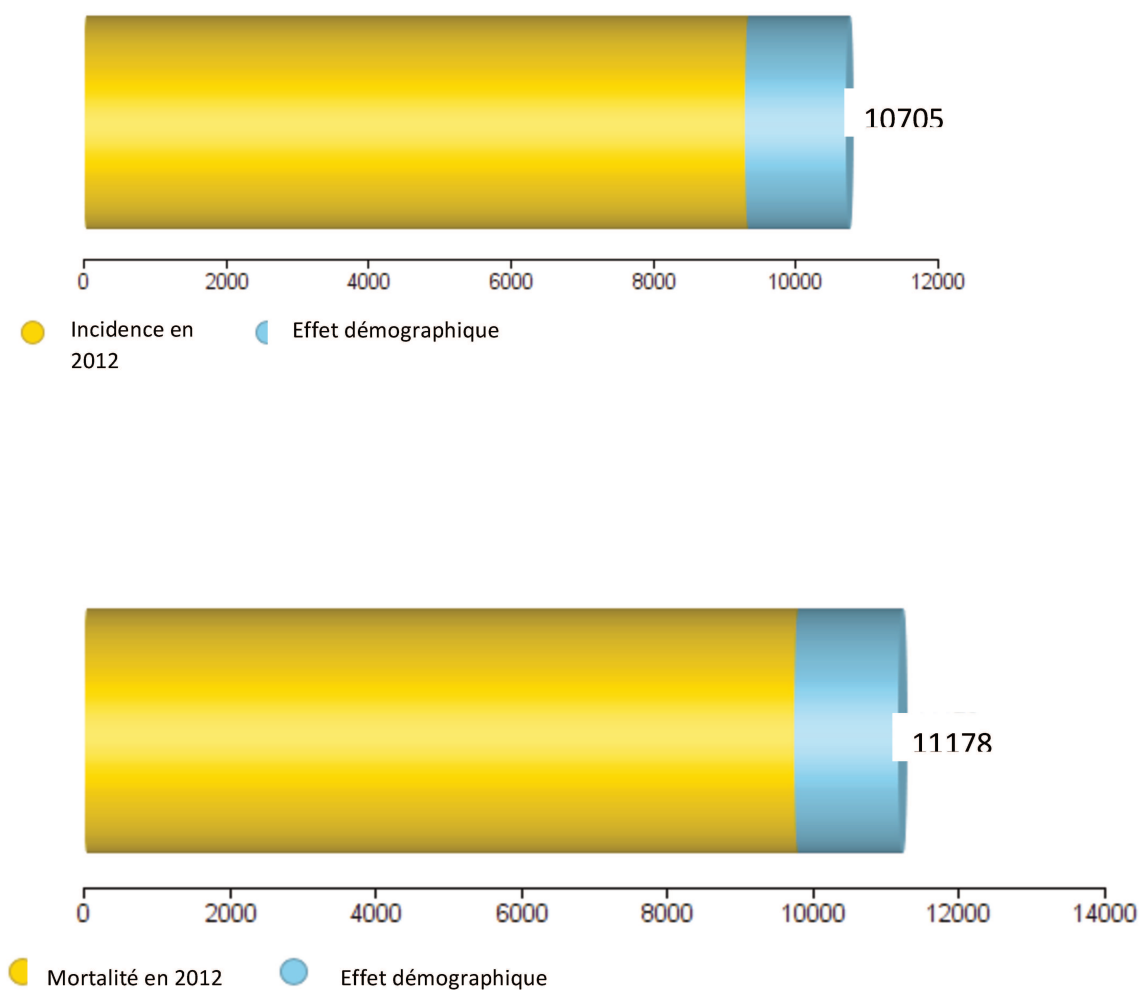
Le cancer du pancréas tel qu'on l'entend est donc l'adénocarcinome canalaire pancréatique (ADCP). Avec une incidence totalisant 10700 nouveaux cas par an en France en 2020, il se place au 10^e rang de tous les cancers confondus. Cette incidence ne cesse de croître avec le temps¹ : elle a rapidement doublé (5000 nouveaux cas/an en France dans les années 90) (**Figures 3, 4**). Ce qui est plus préoccupant, c'est le ratio incidence/mortalité. Il s'agit en effet du seul cancer dont la mortalité est aussi proche voire dépasse l'incidence, avec une mortalité par cancer du pancréas estimée à près de 11200 cas en 2020 en France.

2.3 Diagnostic

Si la maladie peut se révéler dans un tableau bruyant (ictère, prurit, douleurs abdominales), la présentation clinique reste souvent assez pauvre, avec des signes aspécifiques tels que l'asthénie, l'amaigrissement, des douleurs abdominales peu spécifiques. Il s'agit donc d'une maladie dont le diagnostic est souvent fait tardivement. En l'absence d'ictère, ce sont habituellement des douleurs qui amènent à faire une échographie ou un scanner abdomino-pelvien, qui permet de faire le diagnostic du cancer, et d'établir son extension locale (envahissement des vaisseaux mésentériques au contact) et à distance (métastases hépatiques, carcinose péritonéale). C'est l'examen de référence (sensibilité 90%). En cas de doute diagnostique, le scanner peut être complété par une IRM ou par un TEP-scanner, mais surtout par une écho-endoscopie, dont la sensibilité dans les mains d'opérateurs entraînés, atteint 98%². Elle permet également d'apprécier les rapports de la tumeur avec les vaisseaux mésentériques supérieurs, hépatiques et coeliaques. D'autre part, elle permet d'établir un diagnostic formel par la biopsie de la tumeur, avec une sensibilité d'environ 85%, une spécificité de 95%³. Le cathétérisme biliaire permet dans le même temps le drainage biliaire lorsqu'il est indiqué⁴.

La présentation clinique varie donc d'un patient à l'autre, en fonction de la localisation de sa tumeur, mais aussi du stade de sa maladie.

Figure 3 : Incidence et mortalité du cancer du pancréas en France en 2020 (Ferlay *et al.* GLOBOCAN 2012 *International Agency for Research on Cancer* ; 2013)



2.4 Pronostic et survie

Les taux de survie du cancer du pancréas sont globalement très mauvais, passant de 25% à un an, à moins de 5% à 5 ans, tous stades confondus⁵. Si l'on regarde en fonction des stades, les formes métastatiques traitées par chimiothérapie arrivent à une survie de 6 à 12 mois, les formes localement avancées de 12 à 18 mois. La survie des patients opérés (environ 15% de la totalité des patients atteints de cancer du pancréas) est de 20% à 5 ans. Un très faible pourcentage de patients vivra au-delà de 5 ans (longs survivants). Ce groupe de patients n'est à ce jour pas identifiable par des moyens simples et reproductibles, et on connaît mal les facteurs qui seraient à l'origine d'un meilleur pronostic.

2.5. Anatomopathologie

Au sein même de ce type histologique d'« adénocarcinome canalaire pancréatique », on trouve une hétérogénéité inter-tumorale, avec des degrés de différenciation, d'envahissement microvasculaire et périnerveux, un tropisme lymphatique et une propension aux métastases à d'autres organes (foie, poumon, péritoine) très variables d'un patient à l'autre. Cette hétérogénéité inter-tumorale est encore complexifiée par une hétérogénéité intra-tumorale : au sein d'une même tumeur, plusieurs degrés de différenciation peuvent coexister.

2.6. Traitement

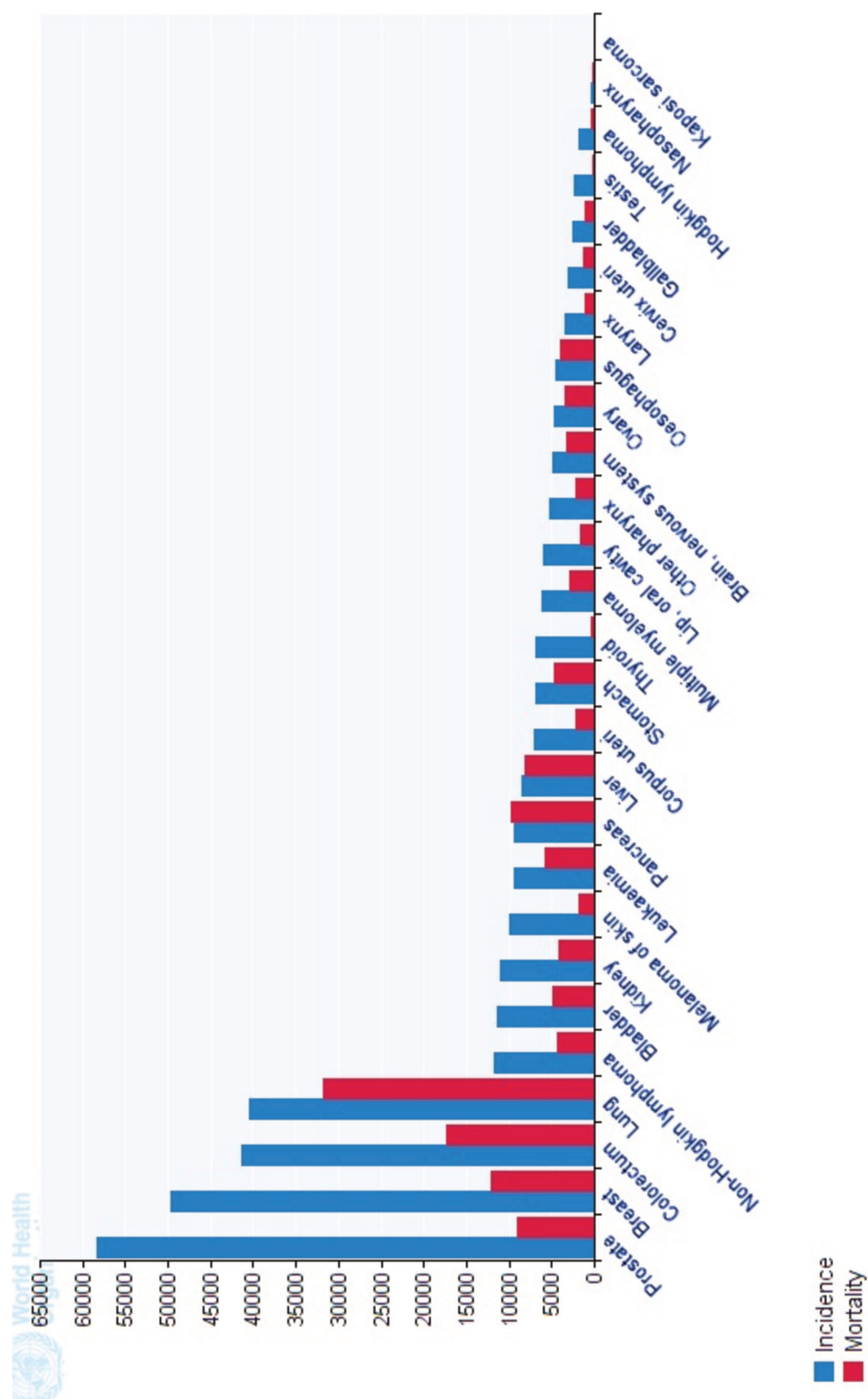
Le traitement du cancer du pancréas va dépendre du stade de la maladie et de l'état général du patient. Il s'articule autour de la chirurgie, de la chimiothérapie, de la radiothérapie, et de traitements médicaux symptomatiques en cas de situation palliative.

La chirurgie va s'adresser aux maladies localisées chez des patients opérables. Cela ne comprend que 10 à 15% des patients. La chimiothérapie, est elle la pierre d'angle du traitement : elle intervient parfois en préopératoire, presque toujours en post-opératoire (sauf si le patient est trop faible pour la supporter), et exclusivement lorsque la maladie est déjà au stade métastatique (60-70% des patients métastatiques d'emblée).

Différentes molécules de chimiothérapie sont utilisées dans l'ADCP.

La gemcitabine est le standard de chimiothérapie dans le cancer du pancréas opéré, c'est à dire en chimiothérapie adjuvante. En effet, elle a détrôné la combinaison 5FU/acide

Figure 4 : Position du cancer du pancréas par rapport aux autres cancers, en terme d'incidence et de mortalité, d'après Ferlay *et al.* GLOBOCAN 2012 *International Agency for Research on Cancer* ;



folinique qui était utilisé jusque là, en montrant son efficacité et sa meilleure tolérance. L'essai CONKO 001 a montré une efficacité de la gemcitabine versus surveillance⁶, et l'essai ESPAC 3⁷ a montré une tendance à une meilleure survie globale en comparaison au schéma antérieur 5FU-acide folinique, avec un p non significatif à 0.39. Cependant, sa meilleure tolérance clinique l'a fait devenir le « gold standard » en traitement adjuvant. La gemcitabine a également obtenu l'Autorisation de Mise sur le Marché dans le cadre de tumeurs localement avancées⁸. Plusieurs associations de la gemcitabine ont été testées: avec l'oxaliplatine⁹ (GEM-OX), avec le cisplatine¹⁰ (GEM-CIS), avec des thérapies ciblées (Erlotinib, Sorafenib, Bevacizumab, Cetuximab), avec des résultats timides, ne permettant pas de changer les recommandations. Plus récemment, l'association *gemcitabine-capécitabine* a été proposée comme le nouveau standard de traitement adjuvant à l'issue de l'essai ESPAC-4, avec une survie globale supérieure à l'administration de gemcitabine seule (atteignant une survie globale médiane de 28 mois), au prix d'une plus grande toxicité¹¹.

La combinaison Folfirinox est le « gold standard » du traitement des tumeurs métastatiques depuis l'étude PRODIGE 4/ACCORD 11 de 2010 portée par Conroy¹². Cette combinaison de chimiothérapie a également été étudiée en traitement néo-adjuvant des tumeurs borderline, avec une survie sans progression significativement meilleure, mais une amélioration de la survie globale non significative¹³. Elle est cependant réservée aux patients en bon état général (statut OMS 0-1)

Malgré toutes ces études, il faut savoir que les tumeurs cancéreuses du pancréas présentent une sensibilité médiocre aux chimiothérapies disponibles. En effet, même si le FOLFIRINOX obtient de meilleurs résultats que la gemcitabine en terme de taux de réponse des tumeurs pancréatiques métastatiques dans l'essai PRODIGE 4, en valeur absolue il n'est que de 31.6% pour le FOLFIRINOX, contre 9.4% pour la gemcitabine seule. Ces chiffres alarmants sont expliqués par une résistance des ADCP aux différentes chimiothérapies. Celle-ci a plusieurs causes :

- un problème d'accessibilité des drogues de chimiothérapies aux cellules cancéreuses, du fait du caractère hypovascularisé de la tumeur

- le stroma péri-tumoral, véritable micro-environnement constitué de cellules fibroblastiques, qui compose 15 à 95% du tissu tumoral, et qui constitue une deuxième barrière physique à l'acheminement de la chimiothérapie vers les cellules cancéreuses à proprement parler
- les propriétés même des cellules tumorales, avec la présence ou non de récepteurs aux chimiothérapies, des voies de signalisation activées ou au contraire réprimées

Il existe probablement une multitude de mécanismes de chimiorésistance, qui sont loin d'avoir été élucidés, malgré le nombre d'articles retrouvés dans la littérature si l'on tape les mots clés « pancreatic cancer » et « chemoresistance » dans PubMed. En ce sens, des études doivent encore être menées pour améliorer la réponse des ADCP aux chimiothérapies. Actuellement, le site clinicaltrials.gov recense 42 études en cours en France portant sur l'ADCP.

Le cancer du pancréas est donc une maladie de plus en plus fréquente, au pronostic mauvais et à la survie courte, dont le diagnostic précoce est difficile du fait de sa présentation variable et peu spécifique, et le traitement peu efficace. Face à ce fléau, plusieurs interrogations sont légitimes : existe-t-il des moyens de prévention, ou des facteurs de risque qui seraient modifiables ou du moins identifiables ? L'hétérogénéité clinique, qui transparaît dans le mode d'apparition, l'anatomopathologie, le pronostic et la survie, est-elle sous-tendue par une hétérogénéité biologique, génétique ou épigénétique ? Peut-on extraire des groupes de tumeurs, ou des groupes de patients, qui seraient homogènes et qui pour lesquels un traitement plus ciblé pourrait être efficace ?

3. LA PREVENTION

La prévention du cancer passe par la connaissance de ses facteurs de risque. En ce qui concerne l'ADCP, il existe des facteurs de risque non spécifiques qui sont aussi ceux d'autres cancers ou maladies cardio-vasculaires. Ceux-ci se potentialisent probablement, et il est difficile d'extraire la part de chacun des différentes études réalisées. Il semble que la plupart des facteurs de risque soient « modifiables » par des règles hygiéno-diététiques qui sont déjà des priorités des politiques de santé publique des pays les plus touchés.

Le *tabagisme* est LE facteur de risque prouvé du cancer du pancréas. Les patients fumeurs auraient 5 à 6 fois plus de risque de développer un cancer du pancréas, et à un âge plus jeune. Le risque de développer un cancer du pancréas dépendrait de la durée du tabagisme, de son intensité, et de l'âge de début¹⁴⁻¹⁶

La *pancréatite chronique* favoriserait également la survenue d'un cancer du pancréas, par des phénomènes d'inflammation chronique de la glande pancréatique¹⁷. Bien que la pancréatite chronique héréditaire soit le meilleur modèle d'étude des relations entre pancréatite chronique et ADCP, les techniques d'analyse moléculaires ont permis de montrer que la plupart des mutations à l'origine de ces pancréatites chroniques n'étaient pas retrouvées dans les cancers du pancréas.

Le *diabète sucré de type II*, conséquence d'une insulino-résistance chez des patients en surpoids ou obèses, est fréquemment associé au risque de cancer du pancréas. Cependant, comme suggéré dans une revue de la littérature à la fois clinique et scientifique par Magruder *et al.* en 2011¹⁸, on peut se demander dans quel sens se fait la relation de cause à effet. Le diabète de type 1 et le diabète de type 2 sont tous deux associés à un risque plus élevé (40 à 100% d'augmentation de risque) d'ADCP à partir de 5 ans d'évolution. Par ailleurs, le traitement du diabète de type 2 et de l'obésité diminuerait ce risque, notamment en cas de traitement par metformine. Mais un troisième type de diabète, le diabète de type 3, a été décrit comme une forme induite par un cancer du pancréas. Il constituerait un groupe à risque au sein des diabétiques de type 2, à hauteur de 30% des patients.

De manière générale, l'*obésité* est un facteur de risque de cancer. Son impact dans la survenue et dans le pronostic dans le cancer du pancréas a été étudié, et il apparaît qu'il s'agit là aussi d'un facteur de risque. Plusieurs études ont montré une corrélation entre IMC et cancer du pancréas^{19,20}. Ces résultats d'études de cohorte sont corroborés par une méta-analyse d'études prospectives publiée en 2007, concluant que le risque relatif de développer un cancer du pancréas augmente de 1.12 tous les 5 points d'IMC²¹.

Aucun facteur protecteur n'a été mis en évidence, que ce soit alimentaire (bien qu'une alimentation de type « crétoise » soit conseillée plutôt qu'une alimentation riche en viande rouge), ou allergique.

Il apparaît clairement qu'aucun facteur de risque ne soit clairement identifié et qu'aucune mesure de prévention spécifique ne soit assez efficace.

4. LES POPULATIONS A RISQUE

L'ADCP ne rentrant pas dans les critères de dépistage de masse (pas assez fréquent, et aucun marqueur biologique spécifique), il paraît utile de cibler certaines populations à risque pour organiser un dépistage individuel.

Ainsi, avoir une histoire familiale de cancer du pancréas expose à un sur-risque : 4.5 fois plus élevé en cas d'un antécédent au 1^{er} degré, 6 fois plus élevé en cas d'antécédent d'ADCP chez 2 parents au 1^{er} degré, jusqu'à 32 fois plus si 3 ou plus de personnes atteintes au 1^{er} degré²². Tous les cas d'agrégation familiale ne s'intègrent pas dans une forme héréditaire. On estime que 4 à 10% des cancers du pancréas s'intègrent dans une histoire familiale d'ADCP, dont 20% seulement dans le cadre d'une mutation constitutionnelle. On a retrouvé 14 mutations associées au cancer du pancréas familial, dont en particulier les gènes BRCA1, BRCA2 (associés aux cancers mammaires et ovariens), MLH1, MSH2, MSH6 (classiquement retrouvés dans le syndrome de Lynch)²³. Le **tableau 1** présente les formes familiales les plus connues. Par ailleurs, il semble exister des mutations sporadiques conduisant à un phénotype « BRCAness », dont l'implication thérapeutique est à l'étude, notamment avec les inhibiteurs de PARP et de ATM/ATR.

Il existe également des lésions précurseurs du cancer du pancréas, au nombre de trois : les PanINs (Pancreatic Intraepithelial-Neoplasia), les TIPMP (Tumeurs Intracanalaires Papillaires et Mucineuses du Pancréas), et les cystadénomes mucineux²⁴.

PanINs ou Pancreatic Intraepithelial Neoplasia

Ce sont des néoplasies pancréatiques intra-épithéliales non invasives microscopiques, non visibles à l'imagerie. Elles évoluent en 3 stades avant de dégénérer en lésions cancéreuses invasives de type adénocarcinome. Ce sont des lésions asymptomatiques, qui sont retrouvées sur l'analyse anatomopathologique de pièces opératoires, juxtaposant des

adénocarcinomes en majorité, mais aussi dans des contextes de pancréatite chronique. Il peut y avoir des lésions de PanINs dans des pancréas sains, mais le plus souvent de bas grade (1 ou 2). Les différents stades de PanINs sont en fait un continuum d'altérations génétiques qui se traduisent par des atypies anatomopathologiques, comme l'explique le « PanINgramme » (**Figure 5**) proposé par Feldmann *et al.* en 2007²⁵, maintes fois repris et complété au fur et à mesure des altérations géniques mises en évidence. L'on peut y voir que la mutation du gène KRAS, rarement retrouvée au sein d'un pancréas normal, est précoce dans la formation des PanINs, et que sa fréquence augmente graduellement entre le stade PanIN I et ADCP pour atteindre plus de 90%.

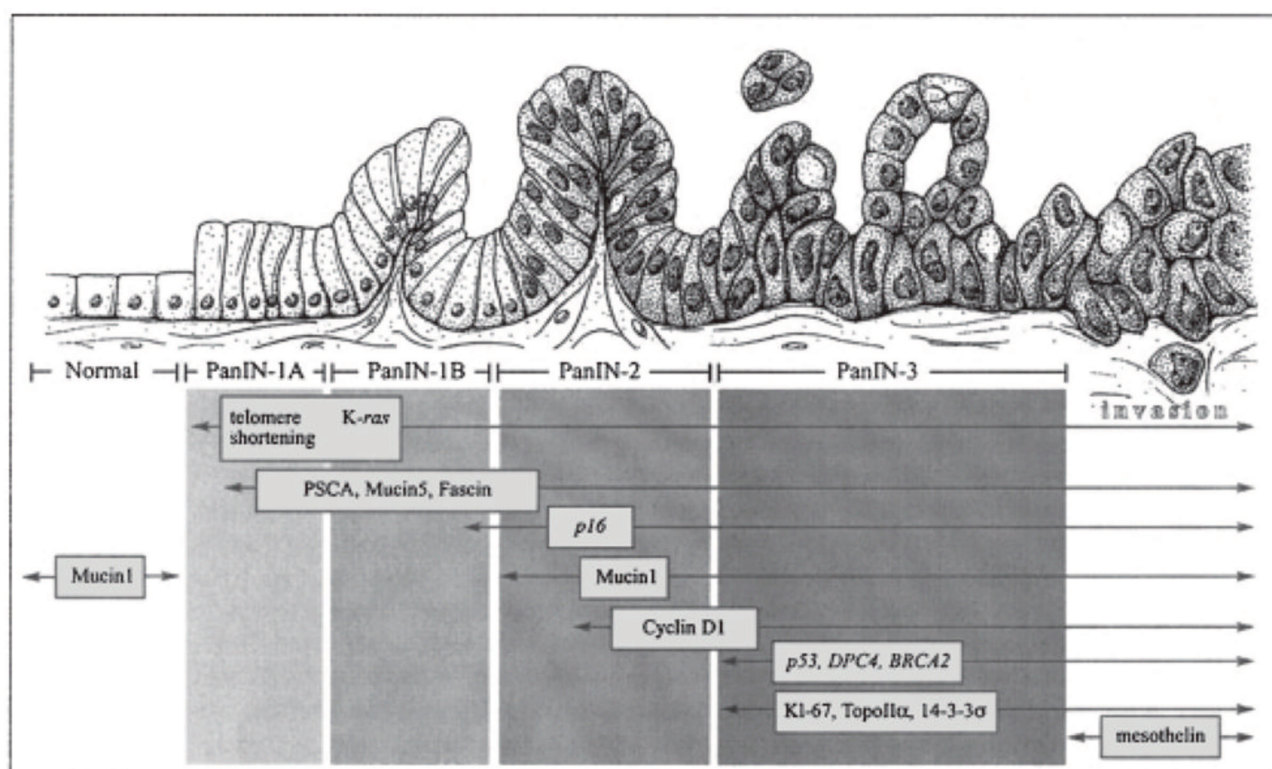
TIPMP ou Tumeurs Intra-Papillaires et Mucineuses du Pancréas.

Il s'agit de lésions mucineuses, intra-épithéliales, développées aux dépens des canaux pancréatiques, formées de papilles tumorales mucosécrétantes, dont le processus d'oncogénèse va comporter plusieurs stades de dysplasie. Quatre sous-types histologiques sont décrits sur pièce opératoire : gastrique, intestinal, pancréto-biliaire, oncocytaire. Les TIPMP représentent 50% des kystes pancréatiques réséqués²⁶, avec une augmentation de fréquence remarquable ces dernières années du fait de la définition de celles-ci à la fin des années 90, et des progrès de l'imagerie. Les risques de dégénérescence sont fonction du type de canal pancréatique atteint. L'atteinte du canal pancréatique principal (canal de Wirsung) avec une dilatation de plus de 10mm, est un facteur de risque de dégénérescence et constitue une indication chirurgicale. Pour les TIPMP des canaux secondaires et pour les dilatations faibles du canal principal, des critères de dégénérescence ont été établis, afin de poser l'indication chirurgicale de groupes à risque, sans pour autant imposer une chirurgie lourde à des patients non concernés par un risque fort de cancérisation²⁷. Du point de vue clinique, il semble que l'adénocarcinome du pancréas développé sur TIPMP soit différent de celui développé de façon « sporadique », les ADCP sur TIPMP semblant plus « indolents », avec des stades plus petits, même en s'affranchissant du biais constitué par la surveillance active de ces lésions²⁸. L'étude génétique et épigénétique des TIPMP est gênée par la difficulté d'en obtenir des lignées cellulaires. Néanmoins, il semble que les mécanismes de formation puis de cancérisation des TIPMP, soient également différents, ce qui pourrait expliquer les comportements cliniques divergents. En effet, alors que les quatre gènes les

Tableau 1 : Syndromes génétiques les plus connus pour intégrer un cancer pancréatique (d'après Ali-Khan Catts et al, Ann Surg Oncol 2016) ²³

Name	Gene	Pancreatic cancer risk until age 70 years (%)	Percentage of pancreatic cancer
Hereditary pancreatitis	<i>PRSS1, SPINK</i>	25–40	–
Peutz–Jeghers syndrome	<i>STK11/LKB1 (19p13.3)</i>	11–36	–
Familial atypical multiple mole melanoma	<i>CDKN2A (9p21.3)</i>	17	–
Familial adenomatous polyposis	<i>APC</i>	<5	–
Hereditary non-polyposis colorectal cancer (Lynch)	<i>MLH1, MSH2, MSH6, PMS2</i>	7	<5
Hereditary breast and ovarian cancer	<i>BRCA1 and BRCA2</i>	2–10	~6

Figure 5 : PanINgramme illustrant la progression des lésions de PanINs vers le cancer infiltrant, ainsi que les altérations génétiques à l'origine de cette progression (Feldmann et al., 2007)



plus mutés dans l'ADCP sont KRAS, TP53, CDKN2A et SMAD4 , les TIPMP connaissent plutôt des mutations GNAS et KRAS²⁹. La mutation du gène GNAS (responsable d'une activation permanente de voies de signalisation intra-cellulaire aboutissant à une augmentation de la prolifération cellulaire), est détectable dans 96% du liquide kystique des TIPMP. Cette mutation est donc probablement à l'origine précoce de la formation des TIPMP. La mutation p53, elle, est présente dans 75% des TIPMP invasives.

Cystadénomes mucineux

Il s'agit là aussi d'une lésion kystique du pancréas sécrétant de la mucine, de revêtement épithélial, avec un stroma de type ovarien. Bien que rare (<5% des tumeurs pancréatiques), il est beaucoup plus fréquemment retrouvé chez la femme (sex ratio 1/20). Son histoire naturelle est mal connue, mais son risque de dégénérescence impose sa résection chirurgicale.

5. HETEROGENEITE BIOLOGIQUE

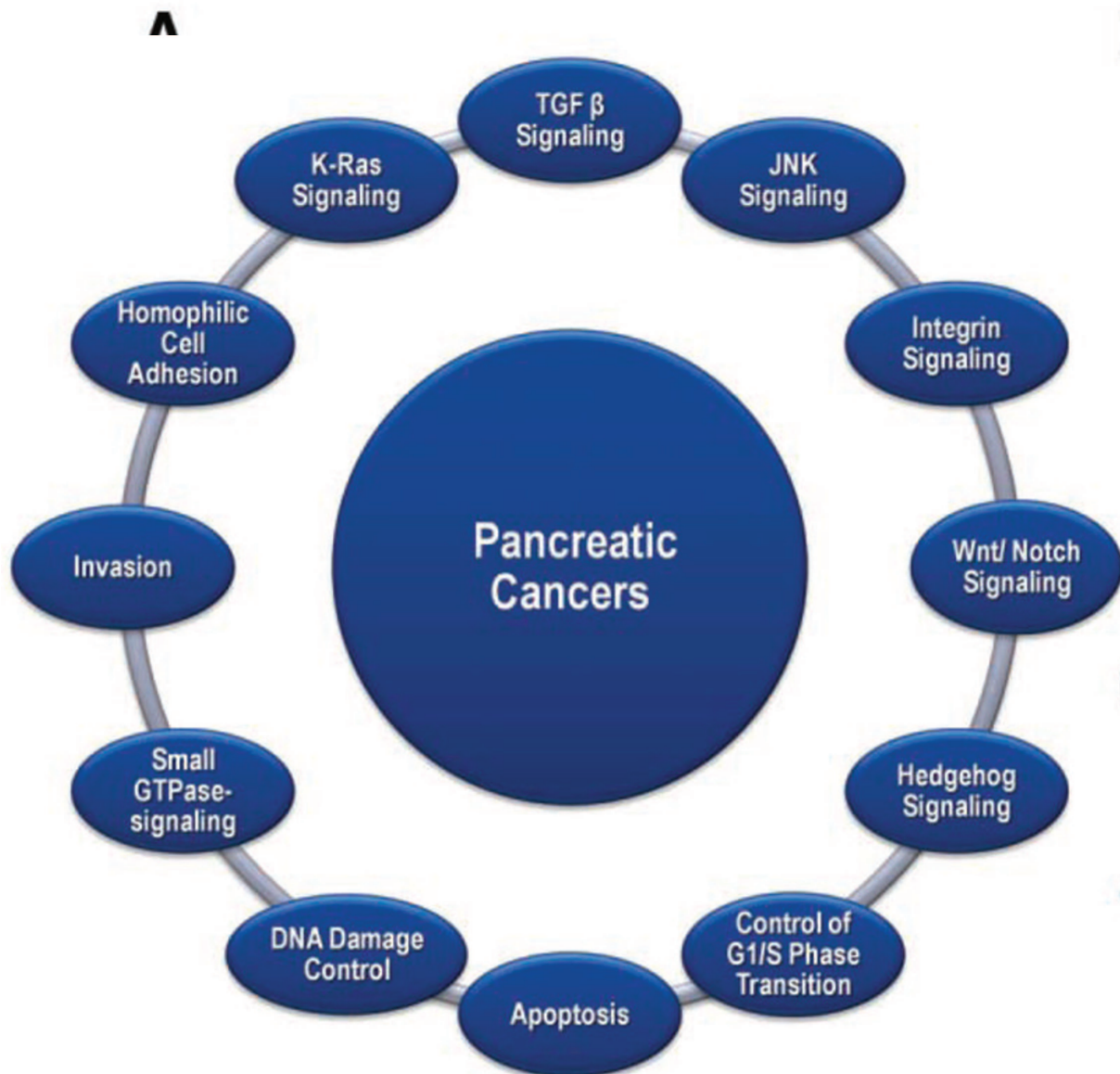
De l'hétérogénéité dans l'histoire naturelle de la maladie, aux différences de pronostics, en passant par l'imprévisibilité de la réponse aux traitements, l'hétérogénéité clinique de l'ADCP existe. Toutes les études cliniques ou pharmacologiques sont menées sur des populations non sélectionnées de patients porteurs d'ADCP, ne les différenciant pas, ou probablement mal, c'est à dire sur des critères qui ne reflètent pas leurs différences (exemple : patients métastatiques, patients avec tumeur localement avancées, etc...) ³⁰⁻³². Les résultats de ces travaux sont alors frustrants : moins de 10% de réponse à la gemcitabine⁸, et à peine un tiers de patients répondeurs au Folfirinox¹². Pour autant, ce sont ces études qui font foi, et qui scellent les recommandations de pratique clinique. Ainsi, le traitement adjuvant standard est la gemcitabine-capécitabine, et la référence pour les patients métastatiques est le Folfirinox, si tant est que l'état général du patient permette d'en supporter les effets secondaires. L'indication du traitement est donc basée, non pas sur le type biologique de la tumeur (puisque l'hétérogénéité biologique n'est pas décryptée), mais sur le stade clinique de la maladie, et l'état général du patient. C'est en ne prenant en compte que la partie superficielle clinique de la maladie, que le traitement a des résultats si

décevants. Il est alors légitime de penser qu'en sélectionnant des groupes de patients plus homogènes, les résultats seront plus pertinents. Mais comment les sélectionner, si ce n'est sur leur présentation clinique ? Ne faut-il pas remonter à l'origine biologique de leur tumeur ? Il est probable que l'amélioration des connaissances du génie biologique de l'ADCP permette de répondre aux différences « phénotypiques » observées, et d'en améliorer la prise en charge diagnostique et thérapeutique.

5.1. Mise en évidence des altérations géniques

En dehors des mutations constitutionnelles que l'on retrouve dans les formes familiales, on dénombre une douzaine de voies métaboliques dont l'altération par des mutations somatiques cette fois, peut aboutir à un processus d'initiation tumorale au sein du parenchyme pancréatique (**Figure 6**). En effet, l'équipe de Jones³³ a démontré en 2008 sur une série de 24 cancers pancréatiques humains, que les cancers pancréatiques contenaient environ 63 altérations génétiques (mutations, délétions, amplifications, ...) qui entraient en jeu dans 12 voies de signalisations différentes, chacune étant altérée dans 67 à 100% des tumeurs. Mais le modèle selon lequel l'ADCP se développerait selon une séquence linéaire de modifications génétiques a été remis en cause, étant en décalage avec la rapidité et l'agressivité variables de l'histoire naturelle de chaque ADCP. Ainsi, Notta *et al.*, ont proposé un concept d'« équilibre ponctué »³⁴ pour expliquer la progression des PanINs vers une tumeur invasive. En effet, l'étude de la variation du nombre de copies d'ADN et des réarrangements géniques associés (polyploïdies et chromothripsis), a montré que le modèle de tumorigénèse ne soit ni graduel, ni ne suive un ordre précis de mutations. Ce nouveau concept est exposé en **Figure 7**. Si les mutations génétiques sont la probable explication de la tumorigénèse, il est plus difficile d'expliquer de manière biologique pourquoi certaines tumeurs vont être plus rapidement métastatiques, ou vont au contraire être plus longtemps localisées, pourquoi certaines vont être plus résistantes à certains traitements, et pourquoi certaines seront *in fine* de meilleur pronostic que d'autres. Il semble que les réponses à ces questions n'aient pas été trouvées dans le génome des tumeurs. En effet, le séquençage des exomes de tumeurs a confirmé les mutations de certains gènes, communes aux ADCP. Mais la comparaison des mutations retrouvées chez des patients long-survivants à celles

Figure 6 : voies de signalisation touchées par des mutations somatiques dans le cancer du pancréas, d'après Jones *et al.*, 2008³³

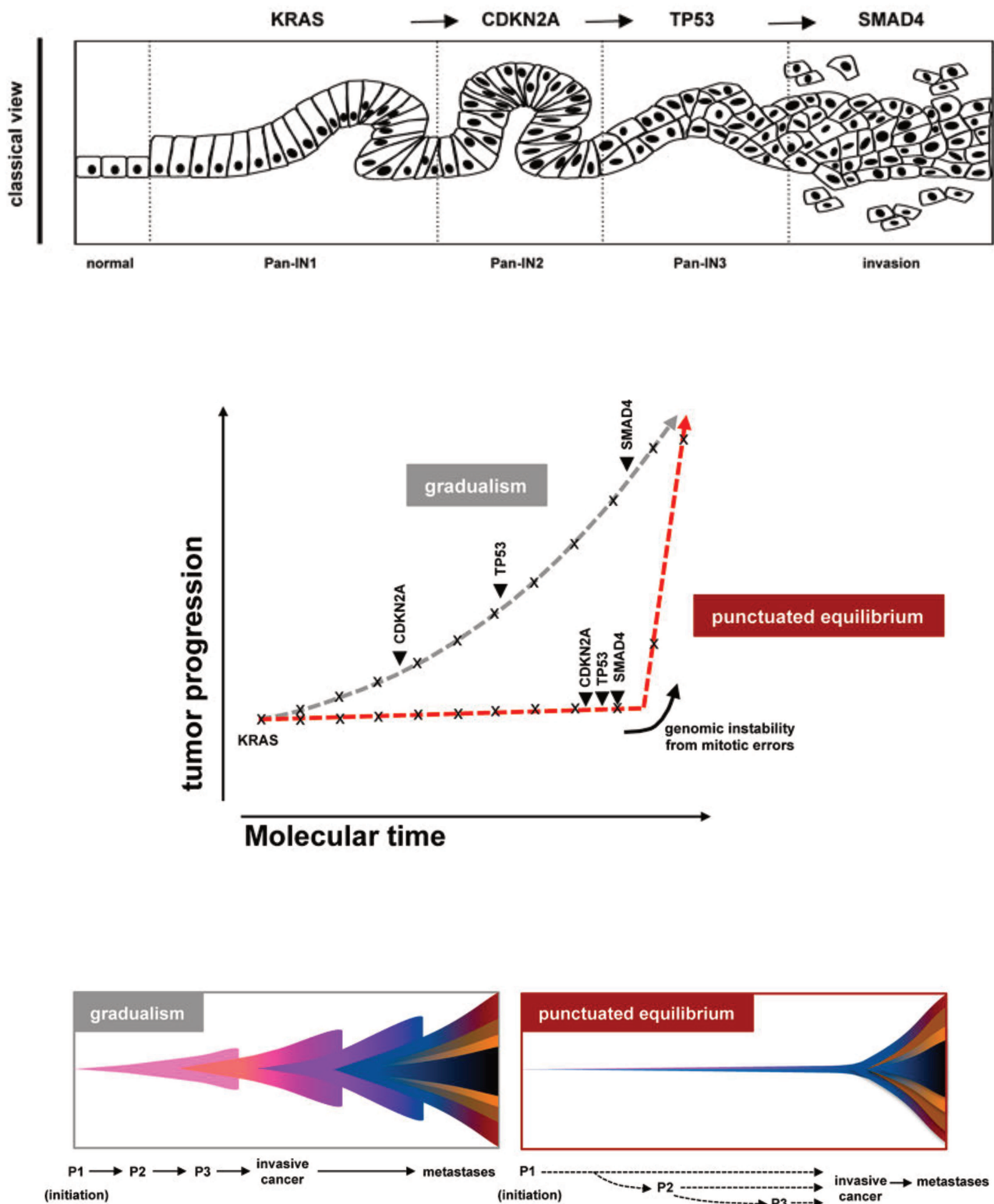


retrouvées chez des patients « standards » atteints d'ADCP, n'a pas retrouvé de différences, suggérant que les mutations somatiques ne pouvaient pas expliquer à elles seules les différences cliniques et biologiques des tumeurs³⁵. D'autre part, si la dérégulation et la mutation de certains gènes cruciaux (KRAS, P53, SMAD4, CDKN2A, MLL3, TGFBR2, ARID1A et SF3B1) ont été identifiées dans l'ADCP, beaucoup ne représentent pas de véritables cibles thérapeutiques. L'accumulation d'altérations génétiques a bien un rôle dans le développement de l'ADCP, mais ne peut pas être la cible de traitements spécifiques. Cette hypothèse est corroborée par des études récentes, comme celle de Witkiewicz *et al.*, menée sur 28 lignées cellulaires dérivées de tumeurs humaines de patients opérés, qui conclue que les analyses géniques ne permettent pas de prédire le profil clinique ni de sélectionner un traitement spécifique³⁶.

5.2 Mise en évidence des altérations transcriptomiques

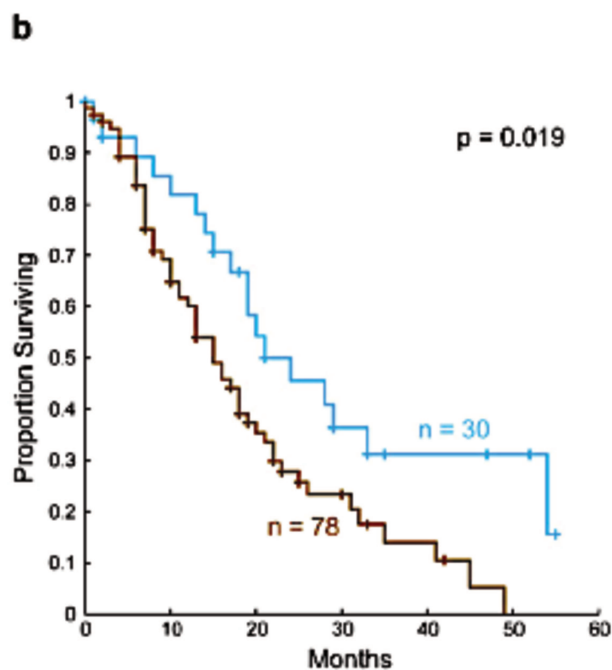
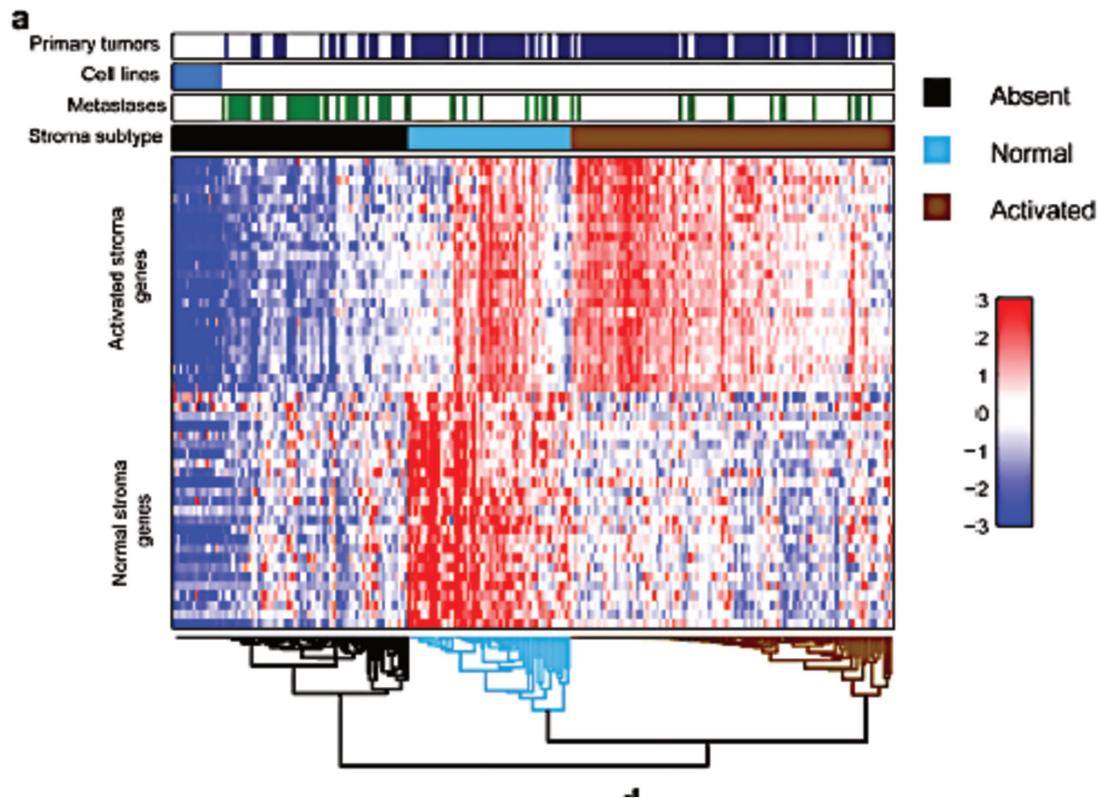
Si les mutations des gènes sont à l'origine des tumeurs, mais ne permettent pas d'en prédire le comportement, c'est qu'il faut chercher en direction de leur expression. Ainsi, l'expression différentielle des gènes entre les tumeurs peut peut-être expliquer les divergences cliniques constatées. Cela est le cas dans les tumeurs de vessie et dans les cancers rénaux à cellules claires, qui sont également réputés pour être très hétérogènes. Leur stratification transcriptomique a récemment permis d'identifier des sous-groupes cliniquement pertinents, et des traitements ciblés efficaces^{37,38}. Ce postulat justifie l'analyse transcriptomique des tumeurs pancréatiques. La difficulté dans le cadre du cancer pancréatique, est d'obtenir des échantillons tumoraux représentatifs de la population de patients atteints d'ADCP. En effet, le recueil de cellules tumorales provenant de patients opérés occulte 85% de patients dont la tumeur est trop avancée pour être accessible à une chirurgie. Les patients porteurs de tumeurs localement avancées ou métastatiques, qui représentent la majorité de la population, ont uniquement, mais obligatoirement, une biopsie tumorale écho-guidée. Il est donc possible d'étudier ces tumeurs, à condition de pouvoir travailler à partir d'un très faible échantillon tumoral. L'autre écueil majeur, est que la tumeur est constituée d'une vaste partie stromale très dense, qui peut masquer l'analyse des processus biologiques spécifiques aux cellules tumorales.

Figure 7 : Modèle de progression tumorale à partir des PanINs, selon le concept de « gradualisme », et concept avancé par l'équipe de Notta³⁴, d'« équilibre ponctué ».



Quelques études ont néanmoins été menées sur le transcriptome de cancers pancréatiques, permettant de définir plusieurs sous-types d'ADCP, homogènes en termes de pronostic et de réponse à certains traitements, et correspondant à des sous-types histologiques³⁹. L'étude princeps⁴⁰ a été celle menée par Collison *et al.*, combinant une microdissection laser à une analyse transcriptomique. Elle a permis de mettre en évidence 3 sous-groupes de tumeurs (classical, quasi-mesenchymal, exocrine-like), dont les pronostics et les réponses thérapeutiques différaient. Ces microdissections virtuelles ont été poussées encore plus loin par l'équipe de Moffitt⁴¹, en caractérisant non seulement la composante tumorale à proprement parler, mais aussi la composante stromale de la tumeur, tout en les corrélant à un pronostic et à une réponse thérapeutique. En effet, ce travail a porté sur l'analyse de l'expression génique de tissu pancréatique normal, de tumeur pancréatique primitive, et de tissu métastatique. Le stroma a pu être divisé en 2 catégories (**Figure 8**) : normal, et activé, correspondant à des populations dont le pronostic était différent : le stroma « normal » correspondant à un meilleur pronostic (médiane de survie 24 mois, survie à 1 an : 82%), et le profil « activé » à un pronostic moins bon (médiane de survie 15 mois, survie à 1 an : 60%). La composante tumorale, a elle pu être classifiée selon 2 types (**Figure 9**) : « classique » et « basal-like » (faisant écho aux tumeurs du sein et de vessie). Là encore, ces 2 sous-types correspondaient à des pronostics différents, la forme classique étant de meilleur pronostic (médiane de survie 19 mois, survie à 1 an : 70%) que la forme basale (médiane de survie 11 mois, survie à 1 an : 44%). Chaque sous-type stromal pouvait se marier avec chaque sous-type tumoral. Par ailleurs, il y avait une corrélation entre réponse au traitement et sous-type tumoral, les tumeurs basales répondant mieux aux chimiothérapies adjuvantes. Les auteurs ont aussi essayé de corréler les sous-types aux altérations géniques des voies métaboliques (TGFβ, RB, NOTCH, CTNNB1, SWI/SNF) et des voies réparations de l'ADN les plus représentées dans le cancer du pancréas, sans trouver aucune association.

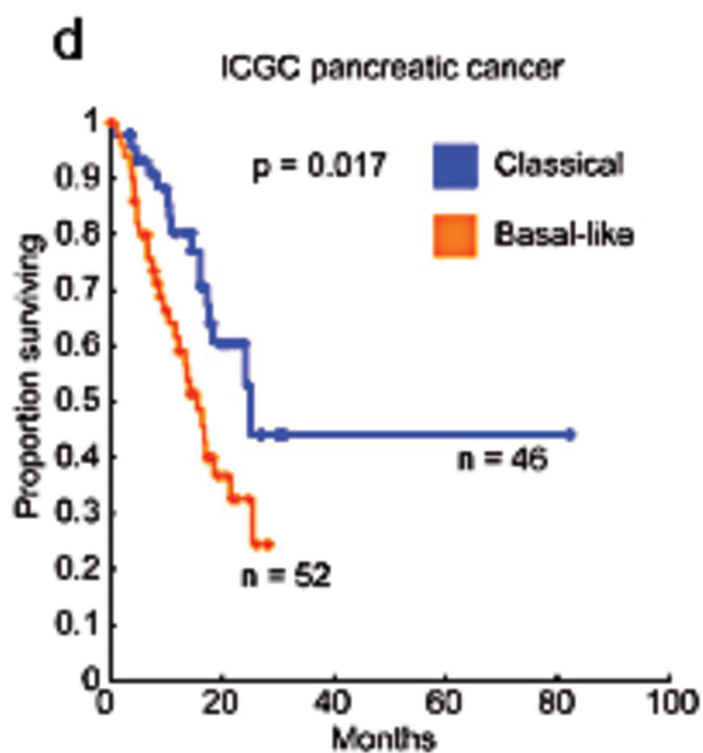
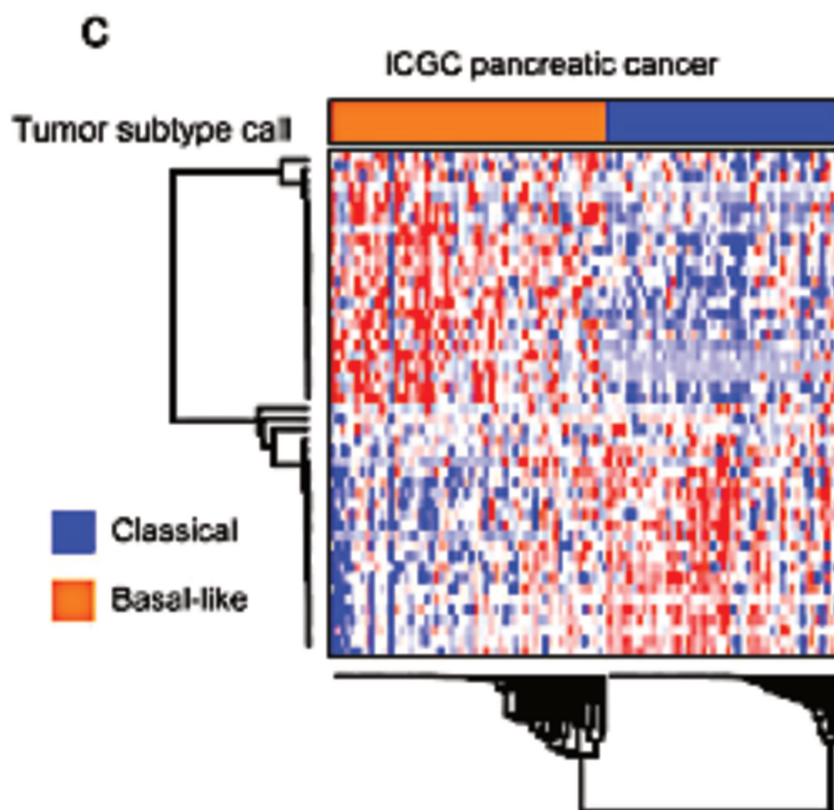
Figure 8 : d'après Moffitt et al⁴¹. a) sous-types de stroma retrouvés par analyse d'expression géniques dans les tumeurs pancréatiques primitives et métastatiques. b) courbes de survie de Kaplan-Meier chez des patients opérés, en fonction du sous-type stromal.



5.3. Mise en évidence des mécanismes épigénétiques

L'épigénétique comprend les procédés qui permettent la régulation de l'expression des gènes sans modification de la séquence ADN. Ces procédés sont néanmoins transmis au cours des divisions cellulaires. Les mécanismes épigénétiques les plus reconnus sont la méthylation de l'ADN, la modification des histones (méthylation, acétylation, phosphorylation, ubiquitination, sumoylation), le modelage de la chromatine, et les ARN non codants (voir **Figure 10**, adaptée de Roy et al.⁴²) L'étude des phénomènes épigénétiques a remarquablement aidé à élucider des aspects de la biologie tumorale que la génétique n'expliquait pas à elle seule. L'accélération récente de la recherche contre le cancer, aidée par les technologies de séquençage nouvelle génération, a permis de décrypter certains mécanismes épigénétiques mis en cause dans les cancers. Dans le cancer du pancréas, des mutations de gènes régulant les mécanismes épigénétiques, ont été mis en évidence. Certains, comme DNMT3A, sont des oncogènes, dont l'expression est donc plutôt augmentée, tandis que d'autres sont des gènes suppresseurs de tumeurs, comme par exemple KDM6A qui a été estimé inactivé dans 18% des cancers pancréatiques. Le **tableau 2**, issu d'une revue de la littérature proposée par Silverman et al, résume les altérations épigénétiques retrouvées dans le cancer du pancréas⁴³. Parmi ces mécanismes épigénétiques entrant en jeu dans les cancers, la méthylation de l'ADN est la mieux caractérisée. La méthylation de l'ADN des promoteurs des gènes suppresseurs de tumeur (comme par exemple APC, BRCA1, p16INK4a) semble être un mécanisme épigénétique majeur de la tumorigenèse. L'équipe de Nones *et al.*, a comparé les profils épigénétiques de 167 ADCP réséqués sans traitement préalable à ceux de 29 pancréas normaux, retrouvant une méthylation différentielle de 3522 gènes, permettant de différencier pancréas non tumoral et ADCP. Cela laisse entrevoir des implications cliniques potentielles, tant en terme pronostique que thérapeutique. Cette méthylation repose sur les DNA methyltransferases, enzymes connues au nombre de 3 : DNMT1, DNMT3A et DNMT3B, dont la surexpression a été rapportée dans le cancer pancréatique. Il a été montré que les niveaux d'expression de ces DNMT augmentaient d'un tissu sain à des lésions précurseurs de PanINs puis d'ADCP, de même qu'en fonction de la taille tumorale, et que l'expression de DNMT1 était corrélée au pronostic de l'ADCP⁴⁴. Il est donc de plus en plus probable que les régulateurs épigénétiques seront les outils diagnostiques, pronostiques, et thérapeutiques de demain.

Figure 9 : d'après Moffitt et al. c) sous-types de tumeur retrouvés par analyse d'expressions géniques dans les tumeurs pancréatiques primitives et métastatiques. b) courbes de survie de Kaplan-Meier, en fonction du sous-type tumoral



6. LE PROJET PaCaOmics (« Pancreatic Cancer et techniques de Omics »)

Jusqu'à très récemment, la caractérisation des tumeurs pancréatiques était étudiée à partir de tissus provenant d'exérèses chirurgicales, soit seulement 15% de patients très sélectionnés. Les résultats robustes concernant la réponse des cellules tumorales pancréatiques aux chimiothérapies provenaient donc d'études réalisées à partir de lignées cellulaires de culture établies (PANC-1, MIA-Paca..). Nous avons émis l'hypothèse, devant l'amplitude de variabilité des réponses cliniques observées chez les patients recevant des chimiothérapies, qu'aucune avancée dans le traitement de cette maladie ne pourrait être faite sans l'étude précise de chaque tumeur. Ainsi, la définition de groupes de tumeurs partageant des caractéristiques communes pourrait augmenter la réponse aux traitements qui, de fait, seraient plus ciblés. Le projet « PaCaOmics » a pour objectif de caractériser les différences existant entre les tumeurs issues de patients dans une population reflétant plus fidèlement la population globale des ADCP, c'est-à-dire les patients opérables, mais également et surtout, les patients présentant une maladie localement avancée non opérable et les patients métastatiques.

6.1. Design du projet

Les buts de ce projet étaient donc :

- d'étudier les différentes caractéristiques moléculaires à la base de l'hétérogénéité tumorale des adénocarcinomes pancréatiques, ainsi que les différences de réponse thérapeutique et de survie des patients,
- d'établir des groupes de patients au sein de l'ensemble des tumeurs évaluées,
- de leur associer des marqueurs moléculaires permettant leur identification,
- de déterminer leur profil de sensibilité aux chimiothérapies,
- de proposer un traitement adapté et personnalisé pour chaque groupe de patients.

Figure 10 : Régulation épigénétique de l'expression génique, selon Roy *et al.*

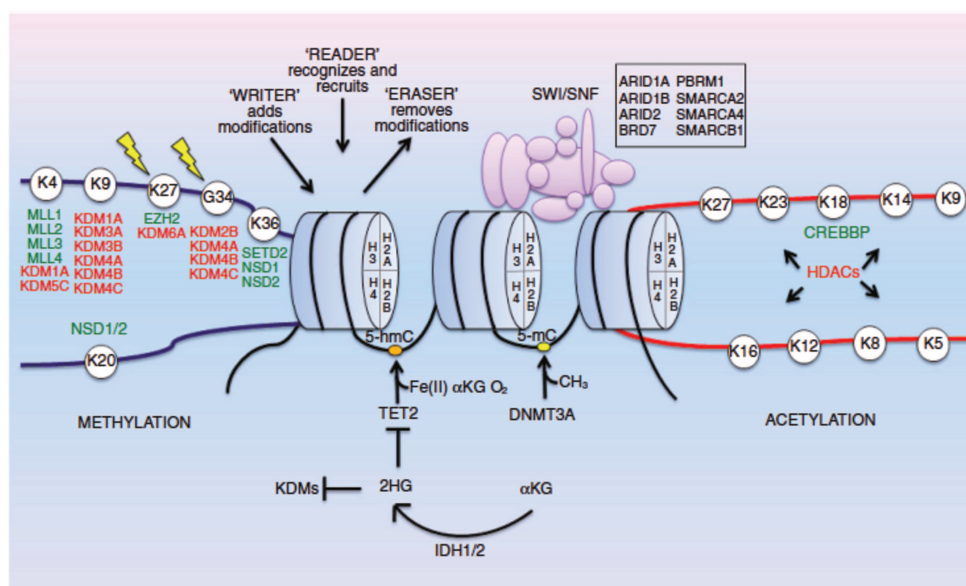


Tableau 2 : Altérations épigénétiques retrouvées dans le cancer du pancréas (d'après Silverman *et al.* ⁴³)

Epigenetic Mechanism	Protein/RNA	Alterations in PDA
DNA methylation	DNMT1, DNMT3A, DNMT3B	Increased expression
Histone modification		
Histone methylation	KDM6A	Loss of copy number, single nucleotide variation, deletion, amplification
	MLL2	Loss of copy number, single nucleotide variation, deletion
	EZH2	Increased expression
	G9a	Increased expression
Histone acetylation	p300	Decreased expression, missense mutation
	HDAC1-3, HDAC7	Increased expression
Histone ubiquitilation	H2AK119Ub1, Ring2, Bim1	Increased expression
Chromatin remodeling	ARID1A, ARID1B, PBRM1, SMARCA2, SMARCA4, ARID2, BRD7	Mutations
	SMARCA2	Promoter polymorphisms, increased expression
	BAF180	Decreased expression
Non coding RNA		
Small RNAs	miR-21, miR-155, miR-196a-2, miR-203, miR-210, miR-222, miR-25, miR-22-3p, miR-642b-3p, miR-885-5p, miR-18a	Increased expression
	miR-101, miR-34a	Decreased expression
lncRNAs	LINC00673	Germline mutation
	H19, uc.345, HOTAIR, HOTTIP, PVT1	Increased expression
	Linc-pint	Decreased expression

Il s'agissait donc, dans un premier temps, de constituer des groupes de patients aux tumeurs semblables au niveau moléculaire, afin de mieux cerner réponses au traitement et pronostics. L'appartenance à un groupe, identifiable de manière simple, reproductible, rapide et valide, serait alors corrélée à un profil de réponse thérapeutique et de survie. De ce fait, les patients pourraient dès le diagnostic, bénéficier d'un projet de soin personnalisé, et éviter les effets secondaires de chimiothérapies qui ne leur seraient pas adaptées. Le bénéfice serait également économique, en évitant d'administrer des produits coûteux dont on n'attend aucune efficacité.

PaCaOmics a débuté en 2011, réunissant 3 centres hospitaliers Marseillais : l'Institut Paoli-Calmettes (promoteur de l'étude), l'Hôpital Nord et l'Hôpital de la Timone, ainsi que le Centre de Recherche en Cancérologie de Marseille (CRCM), plus précisément dans l'unité INSERM 1068, équipe scientifique de stress cellulaire. Afin d'obtenir des cellules pancréatiques humaines viables sur lesquelles travailler, les patients doivent obéir à certains critères d'éligibilité. Dans le cadre de la recherche biomédicale, un consentement libre, éclairé et écrit des personnes participant à l'étude est recueilli, après information par le médecin investigateur en consultation, et après un délai de réflexion suffisant. Par ailleurs, afin de réaliser des analyses de génomique ou de d'épigénomique, le formulaire d'information précise le type de recherche qui est réalisée et le patient a la possibilité d'accepter ou de refuser la conservation de ses échantillons biologiques dans une finalité de recherche scientifique. Les patients éligibles, ayant signé le consentement avec le médecin investigateur, sont enregistrés dans l'étude PaCaOmics après contact avec le promoteur assurant la centralisation des inclusions (Département de la Recherche Clinique et de l'Innovation).

Les patients éligibles sont soumis à 2 méthodes de prélèvements selon le stade de leur maladie:

1. Prélèvement sous écho-endoscopie par ponction à l'aiguille fine chez les patients présentant une maladie d'emblée métastatique ou chez qui un doute sur la résécabilité primaire existe (maladie borderline/localement avancée), subissent une écho-endoscopie au cours de laquelle sont réalisés 2 prélèvements biopsiques, l'un pour l'analyse anatomopathologique diagnostique et l'autre pour l'étude PaCaOmics.

Figure 11 : Fiche d'inclusion au projet PaCaOmics

Bureau d'Etudes Cliniques

☎ : 04.91.22.37.78

☎ : 04.91.22.36.01

✉ : bec@marseille.inclcc.fr



FICHE D'INCLUSION

PACAomics / IPC 2011- 004

Date :

Centre n°

Patient (1^{ère} initiale du prénom, puis 1^{ère} initiale du nom) :

Date de naissance :

Sexe :

Critères à remplir par l'investigateur et à faxer avec le consentement signé au BEC 04.91.22.36.01

Critères d'inclusion		
1 - Suspicion d'ADCP au diagnostic conduisant à une procédure d'écho-endoscopie diagnostique, ou chirurgie d'emblée.		
2 – Patient âgé de plus de 18 ans		
3 – Patient affilié à la sécurité sociale ou bénéficiant d'un tel régime		
4 - Consentement de participation signé		
Critères de non-inclusion		
1 - Personne en situation d'urgence, personne majeure faisant l'objet d'une mesure de protection légale (majeur sous tutelle, curatelle ou sauvegarde de justice), ou hors d'état d'exprimer son consentement.		

Nom de l'Investigateur : Dr

Signature de l'Investigateur :

Nom du technicien de recherche clinique :

<i>Partie réservée au secrétariat du BEC – Institut Paoli-Calmettes</i>
Date d'inclusion
Inclusion conforme
Si non, décision de l'investigateur de maintenir la demande d'inclusion
☞ Accord du promoteur pour inclure
Identification du représentant du promoteur
Date accord

2. Prélèvement tumoral chirurgical chez les patients opérables et dont la tumeur est résécable d'emblée subissent une chirurgie carcinologique pancréatique. Lors de l'analyse anatomopathologique de la tumeur réséquée, un échantillon de tumeur est prélevé et destiné à l'étude PaCaOmics.

Les prélèvements sont ensuite transférés au laboratoire du CRCM, U1068 pour amplification et caractérisation.

6.2. Concept de tumorothèque respirante (Figure 12)

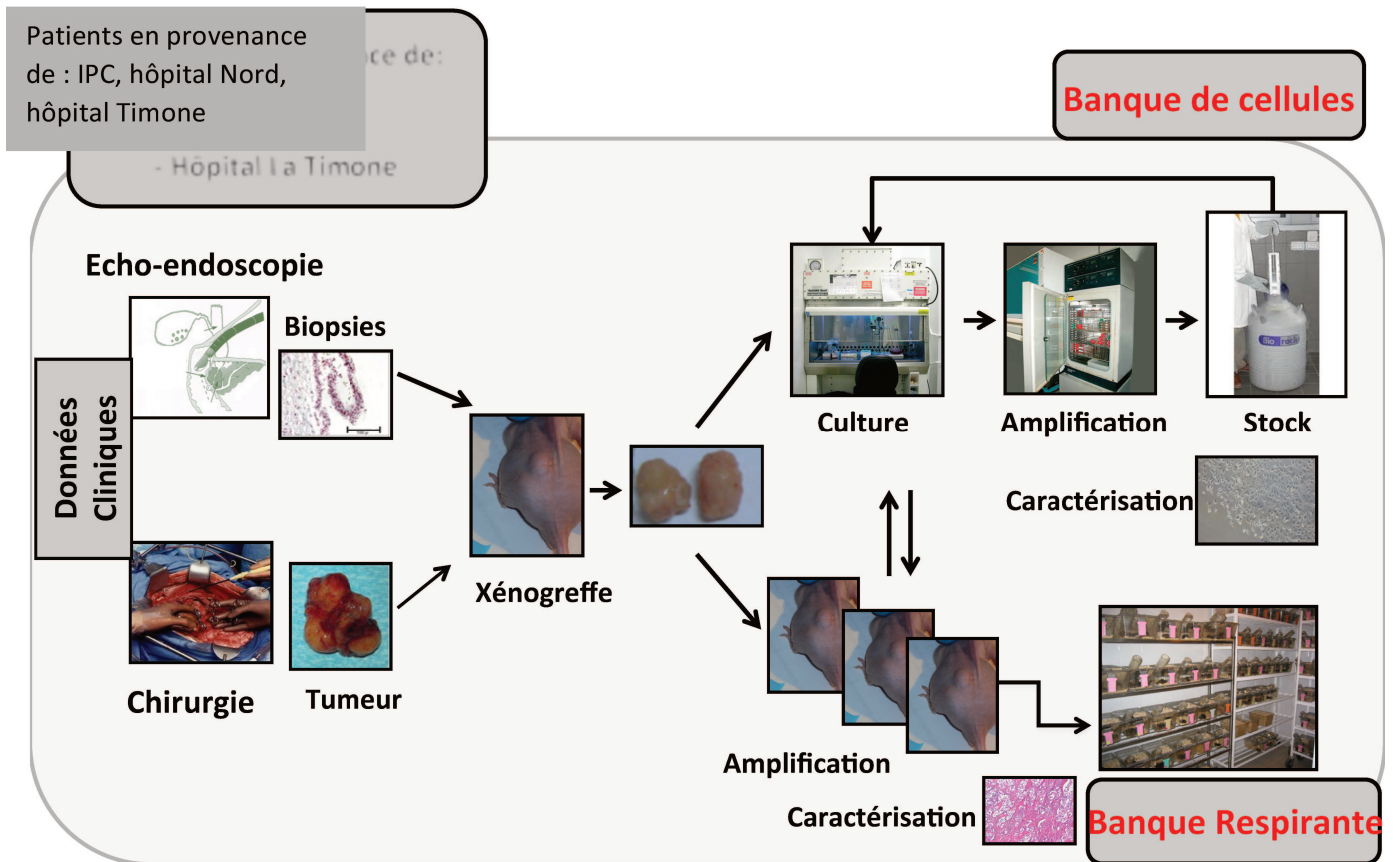
La tumorothèque « respirante » consiste en la greffe sous-cutanée, chez une souris immuno-déficiente, de la tumeur. Ainsi, cette tumeur est conservée « vivante ». On peut imaginer qu'à chaque patient prélevé correspond une souris qui porte une partie de sa tumeur, et qui va permettre d'en étudier toutes les caractéristiques histologiques, moléculaires, génétiques, même après le décès du patient. Chaque tumeur croît jusqu'à atteindre un centimètre cube, volume auquel la souris est sacrifiée, dans le respect des règles d'éthique animale. La tumeur récupérée subit alors plusieurs sorts : une partie est congelée, afin d'être utilisée ultérieurement pour des analyses d'ADN, ARN, protéique, etc... Elle constitue alors une banque « cellulaire ». Une autre est mise en paraffine pour étude histologique. Une troisième est mise en culture cellulaire primaire pour permettre des tests *in vitro*. La dernière partie est réimplantée dans une nouvelle souris immuno-déficiente pour la conserver « respirante », et éventuellement réaliser des tests *in vivo*.

Ces techniques permettent à terme :

- de constituer des bio-banques par congélation des cellules et des xénogreffes,
- mais aussi de maintenir les tumeurs au stade « respirant » par plusieurs passages de xénogreffes dans des souris immuno-déficientes.

A l'aide des xénogreffes et des cultures primaires, l'objectif était de réaliser :

Figure 12 : parcours de la tumeur humaine de son prélèvement sur le patient à son analyse *in vivo*, et concept de tumorothèque respirante.



a. Des techniques de « Omics » à haut débit

b. Des tests de sensibilité aux drogues avec établissement des profils de sensibilité *in vitro* aux chimiothérapies.

Pour chaque patient inclus, sont recueillies prospectivement des données:

- démographiques : âge, sexe, tabagisme;
- cliniques : antécédents familiaux et/ou personnels de cancer, date du diagnostic,
- biologiques : bilirubine totale et conjuguée, marqueurs tumoraux ACE et CA19.9,
- histo-pathologiques : siège de la tumeur, taille tumorale, classification TNM, envahissement ganglionnaire, embols lymphatiques et périnerveux, marges d'exérèse (lorsque chirurgie), métastases à distance;
- thérapeutiques : date de la 1^{ère} cure de chimiothérapie, molécules de chimiothérapie utilisées, durée du traitement, réponses au traitement
- de survie : date de rechute ou de progression, date des dernières nouvelles et enfin date de décès.

Les données expérimentales et cliniques sont ensuite analysées et intégrées par bio-informatique.

Le projet PaCaOmics recensait au 1^{er} octobre 2017, plus de 260 patients inclus.

6.3. Hétérogénéités

Grâce à cette base de données conséquente, nous avons pu confirmer l'hétérogénéité des ADCP :

6.3.1 Hétérogénéité histologique et anatomopathologique

C'est celle que l'on peut voir, autant dans les cultures primaires issues des tumeurs xénogreffées puis recueillies, que dans les blocs de paraffine. Il est intéressant de noter qu'il n'existe quasiment pas de dérive cellulaire anatomopathologique entre la tumeur humaine prélevée lors d'une biopsie ou une intervention chirurgicale, et la tumeur qui croît dans la

souris immunodéficiente, même après plusieurs passages. L'hétérogénéité se révèle dans la différenciation des tumeurs, dans l'organisation et le taux de réplication cellulaire en culture primaire.

6.3.2 Hétérogénéité clinique

Elle se retrouve autant dans la différence de présentation clinique des patients, que dans l'agressivité reflétée par le temps de pousse tumorale au sein d'une souris immunodéprimée.

6.3.3 Hétérogénéité dans la réponse aux traitements

Objectivée par la mesure de résistance et de sensibilité aux différentes chimiothérapies (chimiogrammes) testées : 5-FU, oxaliplatine, irinotécan, gemcitabine, taxotere. Les chimiogrammes, répétés en triplicats, étaient cohérents sur une même lignée cellulaire. On a pu mettre en évidence des lignées plus ou moins sensibles à certaines drogues. Des résistances étaient retrouvées même à des concentrations élevées de produit. A partir de ces constatations, nous avons eu deux buts : premièrement, objectiver, comprendre, et essayer de corrélérer les différents types d'hétérogénéité observés. Pour cela, nous avons réalisé des études multi-omiques à partir des échantillons tumoraux recueillis auprès des patients inclus dans le protocole PaCaOmics. Ce premier axe de recherche a fait l'objet de 2 publications :

- *Transcriptomic Analysis Predicts Survival and Sensitivity to Anticancer Drugs of Patients with a Pancreatic Adenocarcinoma*
- *Novel therapeutic targets for pancreatic adenocarcinoma revealed by tumor-stroma cross-talk analyses in patient-derived xenografts* (en relecture)

Deuxièmement, et parallèlement, nous avons testé des combinaisons thérapeutiques, dans le but de sensibiliser les cellules tumorales aux drogues déjà testées. Pour ce faire, nous avons étudié des molécules déjà existantes (et déjà utilisées en pratique clinique pour d'autres pathologies), dont l'effet pourrait interagir avec le métabolisme de la cellule cancéreuse pancréatique, en testant l'effet synergique avec les chimiothérapies

communément administrées. Nous nous sommes également attachés à rechercher des marqueurs biologiques prédictifs de la potentialisation d'une chimiothérapie par une autre molécule, afin de cibler les tumeurs, et donc les patients, qui pourraient en bénéficier. Ce deuxième axe de recherche a également fait l'objet de 2 publications :

- *A subgroup of pancreatic adenocarcinoma is sensitive to the 5-aza-dC DNA methyltransferase inhibitor*
- *A pancreatic ductal adenocarcinoma subpopulation is sensitive to FK866, an inhibitor of NAMPT*

Ces deux axes de recherche sont développés de manière concomitante et se complètent. Ils seront tour à tour abordés dans ce travail de thèse.

1. Premier axe de recherche : l'étude multi-omique au service du décryptage de l'hétérogénéité de l'ADCP.

A partir des tumeurs recueillies au sein du protocole PaCaOmics, nous avons pu réaliser des analyses sur diverses tumeurs : des tumeurs faisant l'objet de biopsies car non accessibles à un traitement chirurgical, et des tumeurs opérées, dont on pourrait penser, intuitivement, qu'elles sont moins agressives. En effet, l'analyse tumorale à partir de xénogreffes issues de patients (Patients Derived Xenografts, PDX), semble être le modèle d'étude disponible le plus proche de la tumeur humaine. Elle a été de plus en plus développée ces dernières années, permettant non seulement d'étudier les cellules *in vivo*, mais aussi leur comportement et leur interaction avec leur environnement tumoral, ainsi que leurs réactions en présence d'agent pharmaceutiques, se rapprochant ainsi d'essais cliniques de phase II^{45,46}.

L'étude de ces tumeurs a porté d'abord sur leur aspect microscopique histologique: ainsi, les cellules ont été mises en culture, ce qui a permis d'en étudier la dynamique (temps de doublement cellulaire), mais aussi leur réponse aux chimiothérapies, lors de la réalisation de chimiogrammes. L'analyse anatomopathologique a été possible après inclusion en paraffine de tumeurs issues de xénogreffes de tumeurs humaines. Ainsi, l'architecture tumorale et le

degré de différenciation ont pu être classifiées. Ces expérimentations ont pu être menées grâce à une technique très fiable de xénogreffe et de mise en culture cellulaire à partir d'échantillons tumoraux provenant de pièces opératoires et de biopsies transgastriques écho-guidées. L'analyse microscopique descriptive et fonctionnelle, qui a confirmé l'hétérogénéité biologique des tumeurs, a ensuite été complétée par une étude transcriptomique à partir d'ARN, par approche Affimetrix. Ainsi, à partir de quelques PDX, nous avons pu obtenir une analyse transcriptomique complète, puis grâce à des outils bioinformatiques d'analyse non supervisée, mettre en évidence plusieurs aspects. D'une part, la séparation, par la transcriptomique, de 2 groupes de patients selon leur survie. D'autre part, et de manière indépendante, la prédiction d'une sensibilité ou d'une résistance à différentes chimiothérapies communément utilisées dans l'ADCP. Ces conclusions étaient tirées du fait d'une expression différentielle de nombreux gènes, avec une signature moléculaire propre à la survie des patients.

Nous avons ainsi pu montrer des différences d'expressions géniques corrélées à la survie des patients, et à la sensibilité des tumeurs aux drogues, sans lien entre ces deux phénomènes. Ainsi, une signature moléculaire de 944 gènes d'intérêt permettait de prédire si un patient allait être « survivant à court terme » (moins de 8 mois de survie) ou « survivant à long terme » (plus de 8 mois de survie). Comme attendu, la plupart des tumeurs issues de biopsies (patients dont la tumeur était non résécable), étaient dans le groupe de patients de plus mauvais pronostic. Mais ce groupe de courts survivants intégraient également des patients qui avaient été opérés. Il s'agit d'une notion très importante pour les cliniciens. En effet, la prise en charge d'un patient atteint d'ADCP, se base sur des « macro » critères cliniques, radiologiques, et biologiques, et des « micro » critères anatomopathologiques. Mais pas, du moins pas encore, des critères moléculaires. Ce résultat nous laisse penser qu'au delà de la présentation clinique, qui ne représente que la partie émergée de l'iceberg, le clinicien doit pouvoir avoir accès à la biologie tumorale, pour véritablement soigner le patient dans un but curatif. Ainsi, tumeur résécable et tumeur métastatique pourrait être 2 stades d'une même maladie, prise à deux temps différents, mais aussi 2 maladies d'agressivités différentes. De même, deux patients métastatiques pourraient avoir deux biologies tumorales différentes, l'une agressive serait très rapidement mortelle, quand l'autre resterait stable plus longtemps, même à un stade avancé. La corrélation de la survie à l'analyse transcriptomique offre donc bien des perspectives, qui dépassent des principes de médecine de base. Cela permet de se poser la question suivante : ne devra-t-on pas un jour se baser plutôt sur la biologie de la tumeur, que sur sa présentation clinique ? Ne devons-nous pas opérer des patients métastatiques de tumeurs de bons pronostics, et traiter de manière palliative des patients dont une tumeur est résécable mais dont on sait que l'évolution sera rapidement fatale ? Le deuxième résultat était une signature moléculaire permettant de prédire la sensibilité aux drogues des tumeurs. Il est intéressant de souligner que la sensibilité d'une tumeur à une chimiothérapie est indépendante de sa sensibilité à une autre chimiothérapie, mais aussi que les gènes qui la régissent ne sont pas les mêmes que ceux qui sont exprimés dans la différence de survie. Autrement dit, le pronostic d'un ADCP ne dépend pas que de sa sensibilité aux drogues qui vont lui être administrées. Ces résultats, à tempérer par le faible échantillon (17 patients), ouvraient de belles perspectives dans le domaine de la médecine personnalisée, « à la carte ». L'autre limite de cette étude, est l'abstraction du microenvironnement tumoral, au profit de l'étude des cellules

tumorales *stricto sensu*. Pourtant, certaines équipes, dont celle de Moffitt, ont étudié cellules tumorales, cellules stromales, et cellules normales, lors de « microdissections virtuelles »^{39,41} à partir des profils d'expression génique des cellules. Plusieurs sous-types de cancer du pancréas ont été dégagés de ces études, qui ont permis de distinguer des sous-types de tumeurs pancréatiques. Bien que chaque équipe propose une classification en sous-groupes différente, il en ressort à chaque fois un sous-type de mauvais pronostic, avec des tumeurs très peu différenciées. Nous avons donc voulu nous intéresser au stroma, et particulièrement aux dialogues entre cellules tumorales et stromales. Pour ce faire, nous avons comparé l'analyse transcriptomique de 29 PDX, aux signatures connues et publiées de « microdissections virtuelles ». En effet, le travail sur des PDX, et particulièrement en appliquant notre méthodologie de Simultaneous MApping for Patient-derived xenograft (SMAP), offre l'immense avantage de pouvoir à la fois distinguer et étudier les interactions entre tumeur et stroma *in silico*. Nous avons donc effectué des analyses non-supervisées multi-omiques (ARNm codant pour des protéines, ARN non codant, microARN, méthylations des CpG) sur des tumeurs humaines et des stromas murins. Nous avons d'abord confirmé à nouveau l'hétérogénéité des tumeurs, mais aussi des stromas, avons confirmé que les propriétés génomiques globales (instabilité chromosomique, taux de mutations), ainsi que la variation du nombre de copies, n'avaient aucun lien avec les différents sous-types retrouvés après analyse transcriptomique. Nos analyses étaient concordantes avec la littérature, et nous avons retrouvé les 2 sous-types tumoraux déjà décrits (classical et basal). Nous avons également mis en évidence un sous groupe de tumeurs au sein des cancers « classical », qui sont CIMP (CpG Island Methylator Phenotype). Aucune différence d'expression génique n'a pu être mise en évidence entre le sous-groupe CIMP et les autres tumeurs de type « classical » (simplement une sous-expression de certains gènes présente dans toutes les tumeurs « classical » en rapport aux tumeurs « basales »), suggérant que la méthylation des îlots CpG est un des nombreux mécanismes de silence génique qu'il reste à découvrir au sein des tumeurs « classical ».

Ce travail confirme le régissement par des mécanismes transcriptionnels plutôt que d'altérations géniques, du phénotype tumoral notamment en ce qui concerne agressivité de la maladie, profil clinique, et réponse thérapeutique. Il entr'aperçoit également la méthylation des îlots CpG comme un des processus phare de la régulation d'expression génique au sein des tumeurs. Cela offre des perspectives thérapeutiques qui jusque là étaient des impasses, tant les altérations géniques étaient des cibles difficilement atteignables. En effet, les dérégulations épigénétiques des voies métaboliques semblent plus accessibles à un traitement. Dans notre travail, les dérégulations du métabolisme cholestérolique ressortent de manière importante. Il a d'ailleurs été décrit dans la littérature, une association entre métabolisme du cholestérol, et cancer du pancréas^{47,48}, et les traitements classiques de l'hypercholestérolémie (les statines par exemple), sont de plus en plus étudiés, non seulement pour leur impact dans la survie des patients atteints de cancer du pancréas, mais aussi pour leur effet anti-tumoral via des mécanismes épigénétiques, dans l'ADCP.⁴⁹⁻⁵¹ A partir de notre analyse multiomique, nous avons sélectionné le gène NPC1L1, intervenant dans le métabolisme du cholestérol, dont la régulation est très différente tantôt que l'on se trouve dans une tumeur de type « basal », où il est peu exprimé, ou au contraire dans une tumeur de type « classical », dans laquelle il sera surexprimé. Il existe un inhibiteur-compétiteur connu de NPC1L1 : l'ezetimib. Nous avons pu montrer que les tumeurs étaient plus ou moins sensibles à l'ezetimib en fonction de l'expression de NPC1L1, et nous avons également montré que l'effet de l'ezetimib sur les tumeurs organisées en sphéroïdes et en organoïdes était significatif en terme de croissance et de volume tumoral. Car c'est finalement les implications thérapeutiques qui sont les plus importantes à étudier, afin de pouvoir montrer une pertinence en pratique clinique. L'impact de drogues déjà connues et utilisées à d'autres fins a déjà été étudié pour le cancer du pancréas comme pour d'autres tumeurs, soit en monothérapie, soit le plus souvent de façon concomittante avec la chimiothérapie de référence. En effet, le fait d'utiliser des molécules déjà employées dans d'autres indications, permet de connaître à l'avance leur toxicité, leur biodisponibilité ainsi que leur pharmacocinétique, permettant de se passer de phase pré-clinique pour d'emblée tester leur non-infériorité en concomittance avec le traitement de référence. Il s'agit là de notre 2^e axe de recherche.

2. Deuxième axe de recherche : comment améliorer la prise en charge thérapeutique des patients par le biais des résultats de laboratoire : *from bench to bedside*.

Le but ultime du décryptage de l'hétérogénéité de l'ADCP, est de pouvoir cibler les thérapeutiques, pour être curatif et donner les traitements les plus pertinents aux patients, et pour éviter des effets secondaires de traitements inefficaces. Si tous les mécanismes de cryptage ne sont pas encore connus (modifications transcriptionnelles, épigénétiques telles que méthylation), certains ont déjà été repérés et peuvent être ciblés. C'est le cas des DNA methyl transférase (DNMT), enzymes catalyseurs de la méthylation en 5'C des résidus cytosine. Plusieurs inhibiteurs de DNMT ont été développés dans le but d'induire un effet anti-cancéreux par la régulation de l'expression de gènes impliqués dans le cycle cellulaire, la différenciation cellulaire et l'angiogenèse tumorale, par exemple. Les inhibiteurs de DNMT forment donc une classe pharmaceutique prometteuse dans la recherche contre le cancer. Un de ces inhibiteurs, le 5-aza-dC, est déjà utilisé dans le cadre de leucémie myéloïde aiguë, mais a montré très peu d'effet dans les tumeurs solides au sein d'études sur des populations non sélectionnées⁵². Nous avons voulu tester cette drogue sur des tumeurs provenant de PDX, et surtout rechercher un marqueur biologique prédictif de l'efficacité de ce traitement. En effet, en pratique clinique, la sélection de patients potentiellement répondeurs au traitement est essentielle, toujours dans l'optique de personnaliser les thérapeutiques.

Ces résultats sont la preuve que l'on peut sélectionner des patients répondeurs à une thérapeutique, grâce au degré de différenciation de leur tumeur. Cela corrobore parfaitement ce qui est déjà connu depuis longtemps, à savoir le meilleur pronostic des tumeurs bien ou moyennement différenciées par rapport aux tumeurs moins bien différenciées, et également ce qui est en train d'apparaître dans le paysage thérapeutique : la régulation épigénétique de l'expression génique peut être modifiée et constituer une piste thérapeutique majeure. Il n'en reste pas moins que proposer un nouveau traitement est toujours délicat, même quand le traitement de référence est très peu efficace. Nous avons donc également cherché des molécules capables de potentialiser des chimiothérapies déjà utilisées en pratique clinique. En effet, la combinaison de molécules permet d'en potentialiser les effets, voire même d'en réduire les doses et les effets secondaires. Un des meilleurs exemples est le Folfirinox, combinaisons de 5FU, oxaliplatine, irinotecan, qui a permis une avancée spectaculaire dans le traitement des patients métastatiques, et qui va probablement devenir le standard dans le traitement néo-adjuvant des cancers du pancréas. Nous avons voulu cibler le métabolisme tumoral et plus préférentiellement les enzymes clés responsables de l'équilibre NADH/NAD^+ dans la cellule pancréatique tumorale. Elles sont au nombre de 2 : NAMPT (Nicotinamide Phosphoribosyl Transferase) et NMNAT1 (Nicotinamide Mononucleotide Adenylyl Transferase). Nous avons donc testé le FK-866, inhibiteur non-compétitif très spécifique de NAMPT, qui a montré son efficacité dans des essais précliniques⁵³, sans pour autant être responsable de résultats significatifs dans des essais cliniques, lorsqu'il était administré seul^{54,55}. Le FK-866 a été testé avec succès en combinaison avec d'autres traitements dans le cancer gastrique et dans le neuroblastome. Dans notre travail, nous avons encore une fois pu observer l'hétérogénéité des réponses au traitement par FK-866, avec des lignées cellulaires issues de PDX très sensibles, et d'autres très résistantes. Nous avons ensuite testé les combinaisons FK-866 + autres chimiothérapies (gemcitabine, 5-FU, oxaliplatine) chez les lignées tumorales les plus sensibles au FK-866 seul, et de façon surprenante, nous avons trouvé des sensibilités différentes en fonction de la molécule standard combinée.

Ce travail montre les possibilités de combinaison de drogues conventionnelles avec des molécules qui à elles seules ne suffiraient pas à avoir un effet anti-tumoral significatif, mais qui permettent la potentialisation des effets des chimiothérapies. Encore une fois, nous avons pu trouver une corrélation biologique à la sensibilité au traitement, en mettant en évidence une expression du gène NAMPT proportionnelle à la résistance au traitement par FK-866. Ceci est très intéressant, et nous fait penser que dans un futur proche, nous pourrons connaître à l'avance le traitement qui sera le plus efficace pour une tumeur donnée.

Discussion

L'hétérogénéité des cancers du pancréas est un concept qui n'est aujourd'hui plus à prouver. Cependant, il est à développer, investiguer, décrypter. Nos travaux ont permis un certain nombre d'éclaircissements dans ce domaine. Nous supportons l'hypothèse selon laquelle les altérations géniques, mutations, délétions, amplifications, sont des mécanismes à l'origine de l'initiation et du développement tumoral de l'ADCP. Cependant, comme cela a aussi été évoqué pour d'autres cancers hétérogènes comme le cancer du sein et le cancer de vessie, nous pensons que l'hétérogénéité phénotypique, qui nous concerne puisqu'elle se traduit dans le profil clinique, l'histoire naturelle de la maladie, la réponse au traitement et le pronostic de survie, est intimement liée à des mécanismes transcriptionnels. Parmi ceux-ci, les mécanismes épigénétiques comme la méthylation d'ilôts CpG tiennent une place non négligeable, comme l'a montré la superposition des transcriptomes et méthylomes dans notre travail. Ces mécanismes transcriptionnels concernent non seulement les cellules tumorales à proprement parler, mais aussi les cellules constituant le stroma tumoral, son microenvironnement. Ainsi, le stroma ne constitue plus seulement une barrière physique par sa densité et son hypovascularisation, mais il interagit directement avec les cellules tumorales, de manière à créer un environnement hostile aux molécules thérapeutiques. L'analyse non supervisée de tumeurs et stromas *in silico* grâce à des PDX et selon une technique de SMAP, nous a permis de définir 2 sous-types de tumeurs, « basal » et « classical » et 2 sous-types de stroma, « activé » et « classique ». Cette classification est propre à notre équipe, mais rejoint celles déjà publiées dans la littérature³⁹⁻⁴¹, notre sous-type « basal » englobant le « quasi-mesenchymal » et le « basal-like » déjà rapportés. Nous avons pu montrer, en accord avec la littérature, que ces sous-types correspondaient à des pronostics différents. Par ailleurs, l'analyse transcriptomique a aussi pu permettre de prédire la sensibilité à des chimiothérapies connues. Cela est corroboré par des études récentes niant les liens entre altérations géniques et réponses aux traitements³⁶. La connaissance de tels mécanismes de régulation de réponse aux traitements offre de nouvelles cibles thérapeutiques, plus accessibles que les cibles géniques préalablement testées. En effet, décrypter les voies de signalisation dont les tumeurs sont dépendantes, permettrait de les cibler précisément. C'est notamment ce qui a été fait avec l'oncogène C-Myc. Ce facteur de régulation de la transcription influence l'expression de gènes impliqués dans la croissance

cellulaire, l'apoptose et la prolifération. Il a été prouvé par notre équipe que dans les ADCP, on pouvait distinguer, au niveau transcriptomique, des patients « c-Myc high » et des patients « C-Myc low », avec une signature d'expression différentielle de 16 gènes. L'existence d'inhibiteurs de C-Myc par le biais d'inhibiteurs de leur bromodomaines (BETi), a permis de tester l'inactivation de C-Myc, qui est plus efficace chez les patients aux tumeurs « C-Myc high »⁵⁶. Il s'agit là-encore d'une thérapeutique ciblée, dont l'efficacité peut être prédite par une signature transcriptomique.

Cette approche est également appliquée dans d'autres cancers, et la littérature offre un large panel d'articles concernant l'analyse transcriptomique et la thérapie ciblée dans le cancer. Ainsi, dans le cancer du rein à cellules claires, 4 sous-types ont été décrits grâce à une analyse non supervisée du transcriptome, avec des corrélations pronostiques et en terme de réponse au traitement³⁸. De même dans le cancer de vessie³⁷, du poumon⁵⁷, le glioblastome. Le site clinicaltrials.gov recense 3 essais américains ouverts sur transcriptomique et cancer :

- *Molecular Testing of Cancer by Integrated Genomic, Transcriptomic, and Proteomic Analysis*

- *Clinical Sequencing of Cancer and Tissue Repository: ClinOmics*

et surtout, *Evaluation of the NantHealth GPS Cancer Test in Patients With Advanced Cancers*, un essai prospectif visant à comparer la stratégie thérapeutique habituelle, à celle indiquée par un algorithme à partir des résultats d'un test (NantHealth GPS cancer), qui intègre un séquençage génomique, transcriptomique, et protéomique. Cette comparaison va porter sur des critères cliniques et économiques. Cette étude concerne des patients porteurs de cancers variés, dont: cancer du pancréas non opérable, cancer du sein ou de l'ovaire métastatique, cancer du poumon avancé, cancer du colon métastatique, mélanome métastatique, cancer de prostate métastatique résistant à l'hormonothérapie, cancer de vessie, etc... Les tumeurs neuroendocrines pancréatiques sont également l'objet de ce genre d'étude, avec un essai en cours intitulé *Prospective comprehensive molecular profiling in neuroendocrine tumors (NET-SEQ)*. Il est fort probable que dans un futur proche, l'analyse moléculaire de la tumeur soit la clé du succès du traitement ciblé et personnalisé. Les thérapies « conventionnelles » seront toujours utilisées, mais à meilleur escient, et de nouvelles thérapies, plus ciblées, viendront les compléter ou être exclusives.

Perspectives

Bien que très encourageants, ces résultats restent pour le moment inapplicables en pratique clinique. Bien que les PDX permettent une étude précise de la tumeur et de son stroma, cela ne peut se faire qu'en un minimum de quelques mois, le temps de la croissance de la xénogreffe en souris immunodéprimée, puis de son analyse technique. Nous avons développé une technique d'organoïdes et de sphéroïdes, qui ont plusieurs avantages dont celui de travailler sur un matériel en 3 dimensions, reproduisant une structure architecturale tumorale fidèle, en peu de temps, et à partir d'un échantillon cellulaire réduit. En effet, la constitution d'organoïdes peut se faire à partir d'un échantillon de cellules biospiées, permettant de travailler sur tous les patients atteints d'ADCP. La purification d'ARN à partir d'organoïdes et l'analyse transcriptomique par une technologie de type NanoString se fait alors rapidement, et à un coût acceptable. Le but serait, dans un futur proche, de pouvoir biopsier un patient atteint d'ADCP, de travailler sur ces cellules tumorales et stromales à partir d'une technique de mise en organoïdes, pour pouvoir en faire une analyse transcriptomique, notamment de chimiosensibilité. Ainsi, en quelques semaines voire quelques jours, un traitement personnalisé pourrait débiter. Cette perspective de prise en charge est révolutionnaire : on passe de l'administration d'une chimiothérapie standard indiquée en fonction de l'état général du patient et de sa « catégorie » clinique, à l'adaptation en temps réel d'un traitement fait de chimiothérapie et de thérapie ciblée. Il s'agit là de complètement changer les pratiques cliniques. On peut également imaginer que la durée des chimiothérapies, la réévaluation des tumeurs, se fassent également « à la carte », avec des critères moléculaires très fins et avec une détection beaucoup plus précoce que l'imagerie scannographique actuellement utilisée. De même, l'étude des tumeurs en récurrence pourrait nous aider à adapter le traitement à la pression de sélection appliquée à la tumeur lors d'une première ligne de traitement. Il se peut également, que ces avancées aident les cliniciens à prendre certaines décisions qui seraient aujourd'hui impensables : pourquoi ne pas opérer un patient de sa tumeur et de ses métastases si celles-ci sont résécables, à l'instar du cancer colo-rectal ? Les limites n'étant actuellement pas techniques, mais bien oncologiques, avec un mauvais pronostic associé à des tumeurs métastatiques. Ces résultats nous aideront-ils à prendre en charge des patients métastatiques encore

vivants plusieurs années après le diagnostic ? Ou les patients dont les tumeurs sont « borderline », et pour lesquelles on hésite entre une prise en charge curative et une prise en charge palliative, et dont notre seul allié pour la prise de décision, est actuellement le temps ? Ce sont autant de questions dont l'implication clinique est bien réelle et palpable, que ces avancées nous promettent de résoudre.

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GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Transcriptomic Analysis Predicts Survival and Sensitivity to Anticancer Drugs of Patients with a Pancreatic Adenocarcinoma



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A major impediment to the effective treatment of patients with pancreatic ductal adenocarcinoma (PDAC) is the molecular heterogeneity of this disease, which is reflected in an equally diverse pattern of clinical outcome and in responses to therapies. We developed an efficient strategy in which PDAC samples from 17 consecutive patients were collected by endoscopic ultrasound-guided fine-needle aspiration or surgery and were preserved as breathing tumors by xenografting and as a primary culture of epithelial cells. Transcriptomic analysis was performed from breathing tumors by an Affymetrix approach. We observed significant heterogeneity in the RNA expression profile of tumors. However, the bioinformatic analysis of these data was able to discriminate between patients with long- and short-term survival corresponding to patients with moderately or poorly differentiated PDAC tumors, respectively. Primary culture of cells allowed us to analyze their relative sensitivity to anticancer drugs *in vitro* using a chemogram, similar to the antibiogram for microorganisms, establishing an individual profile of drug sensitivity. As expected, the response was patient dependent. We also found that transcriptomic analysis predicts the sensitivity of cells to the five anticancer drugs most frequently used to treat patients with PDAC. In conclusion, using this approach, we found that transcriptomic analysis could predict the sensitivity to anticancer drugs and the clinical outcome of patients with PDAC. (*Am J Pathol* 2015; 185: 1022–1032; <http://dx.doi.org/10.1016/j.ajpath.2014.11.029>)

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal human malignancies and a major health problem. PDAC causes approximately 230,000 deaths per year worldwide.¹ Despite considerable research efforts in the past 20 years, conventional treatment approaches, including surgery, radiation, chemotherapy, and a combination of these, have limited impact. The prognosis is dismal, with only 20% of patients alive 1 year after diagnosis.¹ Given this scenario, the search for new treatments to counter PDAC progression and thereby increase patient life expectancy has been given high priority. Particularly, future therapeutic agents are expected to be molecularly targeted to specifically affect PDAC cells by targeting their weaknesses while

leaving normal tissues undamaged. Recently, studies that reached phase 3 clinical trials in unselected PDAC populations tested bevacizumab, erlotinib, and axitinib, which are molecularly targeted agents, combined with gemcitabine. These trials did not show robust survival benefits, probably because they were essayed on unselected PDAC populations that were highly heterogeneous.^{2–4} In fact, a major impediment to the effective treatment of PDAC is the

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molecular heterogeneity of the disease, reflected in diverse clinical response patterns to therapy. Hence, the starting point of this work was the heterogeneous evolution observed in patients with PDAC, with survival of 2 to 3 months to 5 years after diagnosis and with a strong difference in susceptibility to classical and novel drugs. This may be explained by the fact that each PDAC has a combination of several modifications in intracellular pathways that may result in variable susceptibility to drugs, metastasis development, and, hence, survival.^{5–7} The treatments proposed to the patients do not take into account this heterogeneity. Yet, the drugs received by patients with PDAC are chosen according to their general performance status and the stage of their disease. No study of the tumor can predict its responsiveness to the treatment or give a prognosis to the disease progression. For example, the objective response rate was 31.6% in multidrug FOLFIRINOX-treated patients and only 9.4% in patients treated with gemcitabine, showing that nearly 70% and 90% of the patients, respectively, did not respond.⁸ Another possibility that explains the resistance to systemic therapies is that PDAC tumors are hypovascularized and very rich in stroma, which can represent 15% to 90% of the tumor mass, resulting in a barrier to drug delivery to the transformed cells.⁹

We hypothesized that the variable sensitivity observed in patients with PDAC is mainly due to the intrinsic molecular characteristics of the cancer cells. To characterize this heterogeneity, we obtained 17 novel human PDAC-derived xenografts and established their corresponding primary cultures. We then characterized the 17 PDAC-derived xenografts at the transcriptomic level. The analysis of these data showed significant heterogeneity. However, the bioinformatic study of transcriptome discriminated that patients with long- and short-term survival corresponded to moderately and poorly differentiated histopathologic phenotypes, respectively. We treated the PDAC-derived primary cell cultures with different doses (0 to 1000 $\mu\text{mol/L}$) of five anticancer drugs and found, as expected, that responses were patient dependent. Using this approach, we also found that the transcriptomic analysis was able to predict the sensitivity of cancer cells to the five anticancer drugs most frequently used in PDAC patients.

Materials and Methods

PDAC Samples

Patients were included in this project under the Paoli Calmettes Institute clinical trial number 2011-A01439-32. Three expert clinical centers collaborated on this project after receiving ethics review board approval. Patient informed consent forms were collected and registered in a central database. The tumor tissue used for xenograft development was deemed excess to that required for the patient's diagnosis. Two types of samples were obtained: endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) biopsy samples from patients with unresectable tumors and tumor tissue samples from patients

undergoing surgery. All the samples were anonymized, and postsurgical anatomopathology reports were provided for specimens from each patient. Histopathologic evaluation was performed on 5- μm hematoxylin and eosin-stained sections of patient tumors and xenografts and examined under a light microscope. These sections were compared with the original human tumor when available.

It is obvious that studies on cancer cells, analyzed on whole surgical specimens, introduce a high background in systemic analysis. Therefore, we optimized the conditions of primary culture for PDAC cells obtained by surgery and by EUS-FNA, a technique systematically used for diagnosis in our hospitals. Each sample obtained from EUS-FNA was mixed with 100 μL of Matrigel (BD Biosciences, Franklin Lakes, NJ) and was injected in the upper right flank of a nude mouse (Swiss Nude Mouse Crl: NU(lco)-Foxn1nu; Charles River Laboratories, Wilmington, MA). Each sample derived from surgery resection was fragmented, mixed with 100 μL of Matrigel, and implanted with a trocar (10 gauge; Innovative Research of America, Sarasota, FL) in the subcutaneous right upper flank of an anesthetized and disinfected mouse. When the tumors reached 1 cm^3 , the mice were sacrificed, and the tumors were removed. Xenografts that failed to develop within 6 months were discontinued. The study on animals was approved by the Animal Facility and Experimental Platform (Scientific and Technological Park of Luminy, Marseille, France).

Xenografted Tumor Processing and Cell Culture

Xenografts obtained from mice were split into several small pieces and used for cell culture, histologic analyses, reimplantation into new mice, and freezing. The fragments designated for cell culture were processed in a biosafety chamber: after fine mincing, they were treated with collagenase type V (ref C9263; Sigma-Aldrich, St. Louis, MO) and trypsin/EDTA (ref 25200-056; Gibco, Life Technologies, Grand Island, NY) and were suspended in Dulbecco's modified Eagle's medium supplemented with 1% w/w penicillin/streptomycin (Gibco, Life Technologies) and 10% fetal bovine serum (Lonza Inc., Walkersville, MD). After centrifugation, cells were resuspended in serum-free ductal media adapted from Schreiber et al¹⁰ at 37°C in a 5% CO_2 incubator. Amplified cells were stored in liquid nitrogen. Cells were weaned from antibiotics for ≥ 48 hours before testing.

Gene Expression Microarrays

For the preparation of RNA samples, xenografted tumors were removed from mice at passage 2 and immediately drawn in a cold 4-mol/L guanidinium thiocyanate solution, followed by protein denaturation and RNA extraction according to Chirgwin's protocol.¹¹ Total RNA (1.0 μg) was reverse transcribed for hybridization to the human oligonucleotide array Human Gene 2.0 (GeneChip; Affymetrix Inc., Santa Clara, CA) as described previously.¹² Arrays were

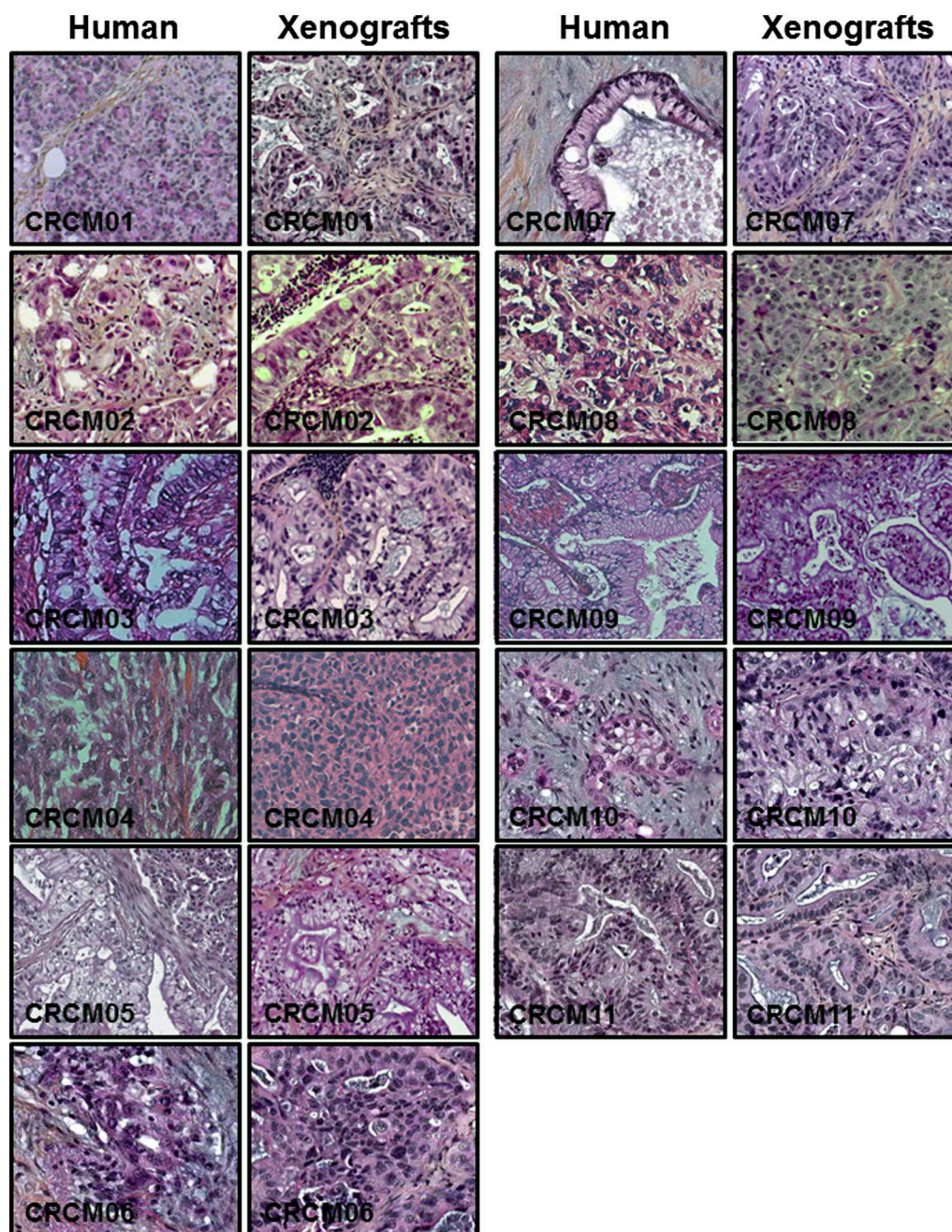


Figure 1 Hematoxylin and eosin staining of pancreatic ductal adenocarcinoma (PDAC) samples from xenografted tumors obtained by surgery and the original PDAC samples obtained by pancreatectomy.

processed using the Affymetrix GeneChip fluidic station 450 (protocol EukGE-WS2v5_450) and were scanned using a GeneChip scanner 3000 G7 (Affymetrix Inc.). GeneChip operating software version 1.4 (Affymetrix Inc.) was used to obtain chip images and for quality control. Background subtraction and normalization of probe set intensities were performed using the method of robust multiarray analysis.¹² Principal component analysis was performed using Genomics Suite software version 6.6 (Partek Inc., St. Louis, MO). Gene set enrichment analysis was performed using the Broad Institute platform, and statistical significance (false discovery

rate) was set at 0.25. Microarray analysis was performed by the CHU de Québec Research Center Gene Expression Platform (Quebec City, Quebec, Canada). All array data are available on the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>; Accession number GSE55513).

Chemograms

Cells were screened for chemosensitivity to five clinically used drugs in patients with PDAC: gemcitabine (Eli Lilly & Co., Indianapolis, IN), 5-fluorouracil (5FU; Teva Pharmaceutical

Industries, Petah Tikva, Israel), oxaliplatin (Hospira, Lake Forest, IL), docetaxel (Sanofi, Bridgewater, NJ), and irinotecan active metabolite named 7-ethyl 10-hydroxycamptothecin or SN-38 (H-0165-10 MG; Sigma-Aldrich). These cells were treated for 72 hours with increasing concentrations of chemotherapeutic drugs ranging from 0 to 1000 $\mu\text{mol/L}$ and from 0 to 100 $\mu\text{mol/L}$ for SN-38. Five thousand cells were plated per well in 96-well plates in serum free defined media. Twenty-four hours later, the media were supplemented with increasing concentrations of drugs and were incubated for an additional 72 hours. Each experiment was performed in triplicate and repeated at least three times. Cell viability was estimated after addition of the PrestoBlue cell viability reagent (Life Technologies) for 3 hours following the protocol provided by the supplier. Cell viability was measured on days 0 and 3 to calculate the replication rate of the cells.

Clinical Outcome

The clinical outcomes of the 17 patients were recorded in a prospective database, and data such as age, sex, medical history, histopathologic stage of differentiation, treatments received (type and duration of chemotherapy), response to chemotherapy, date of progression, metastasis occurrence and localization, and date of death were analyzed. Progression-free and overall survival were then calculated. Short-term survival was arbitrarily defined as a survival time ≤ 8 months, and long-term survival was defined as a survival time > 8 months.

Results

Correlation between Transcriptome and Clinical Outcome

Eleven primary tumors obtained from surgery (Figure 1) and six biopsy samples from EUS-FNA (Figure 2) were implanted subcutaneously into mice and preserved as xenografts by reimplantation. The histopathologic and clinical characteristics of the patients are displayed in Supplemental Table S1. Histologically, all the specimens were PDACs, which were moderately to poorly differentiated. Growth rates to reach a tumor volume of 1 cm^3 ranged from 2 to 6 months. Histopathologic comparisons between patients' primary tumors and xenografts were performed. Hematoxylin and eosin–stained tissue sections showed that xenografts retained the original morphologic features of the human primary tumor (Figure 1). The relative state of differentiation was also retained on passaging until at least six times (data not shown). As is typical of PDAC, a high degree of stroma density was observed in the primary tumors but was progressively lost in xenografts.

Total RNA was obtained from 17 xenografts, and the transcriptome was performed using Affymetrix microchips. The first approach to grouping patients by their expression

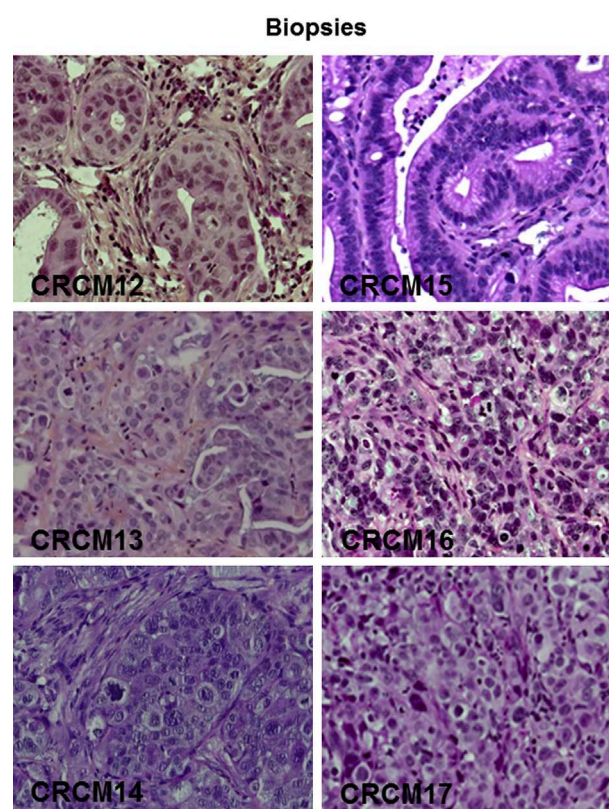


Figure 2 Hematoxylin and eosin staining of pancreatic ductal adenocarcinoma samples from xenografted tumors obtained by endoscopic ultrasound–guided fine-needle aspiration biopsy.

profile used a principal component analysis. We found that the transcriptome from two patient-derived xenografts, CRCM04 and CRCM17, highly detached from the main group of patients (Figure 3A). We analyzed their clinical data and found that both corresponded to two particularly short-term survival patients with poorly differentiated PDAC (Figures 1, 2, and 3, A and B). We then explored the presence of other short-term survival patients with poorly differentiated PDAC in our collection and found that three additional patient-derived xenografts, CRCM16, CRCM08, and CRCM14, corresponded to patients with short survival times of < 8 months (Figure 3B) with poorly differentiated tumors (Figures 1 and 2). We found that their transcriptome result was relatively close to the CRCM04 and CRCM17 ones, indicating a relatively similar expression profile in short-term survival patients with poorly differentiated tumors (Figure 3A). Of the 11 samples obtained from surgery, only 2 corresponded to short-term survivors, whereas of the six samples obtained by EUS-FNA, three corresponded to short-term survivors. In fact, it is not a surprise because samples obtained by EUS-FNA corresponded to patients not suitable for surgery as first-line treatment and, therefore, to tumors that have a worse prognosis.

We next performed a heat map analysis to identify all genes differentially expressed between short- and long-term survival patients. A total of 505 genes appeared as

overexpressed and 439 genes as underexpressed in short-term compared with long-term survivors (Figure 3C and Supplemental Table S2). A Gene Ontology analysis, with a fold change >1.5 and a false discovery rate >0.001, on differential expression showed significant gene enrichment in biological processes associated with cancer, such as cell cycle, mitosis, response to cellular stress, DNA metabolism, chromosome organization, and cellular metabolism (Supplemental Figures S1–S6). Most of these genes were overexpressed in short-term survival with poorly differentiated PDAC, suggesting that these pathways were preferentially activated.

Chemogram

Seventeen PDAC primary cultures were obtained from human PDAC xenografts. Cells were characterized to assess their epithelial origin by immunofluorescence with CK19 antibody, Kras mutation was identified by PCR followed by DNA sequencing, and their transformed status was confirmed by their capacity to produce subcutaneous tumors after injection of 10^7 cells in nude mice. All these primary cell cultures are strongly stained with the anti-CK19 antibody; they are all mutated in codons 12, 13, or 61 and produce tumors in mice within the 8 weeks that follow the injection (data not shown). Fibroblast

contamination was estimated by immunofluorescence using the anti-S100A4 antibody. No more than 5% fibroblast contamination was observed. Cells in primary culture were microscopically analyzed, presenting a high degree of heterogeneity between different patients (Figure 4A). Cell cultures were considered stable after 10 passages and were used during the next 10 passages at maximum to prevent clonal selection.

To study the sensitivity to chemotherapeutic drugs, the cells were submitted to increasing concentrations (from 0.001 to 1000 $\mu\text{mol/L}$) of a set of anticancer drugs and their sensitivity measured to obtain a dose-response curve characterizing each patient. Using this approach we were able to estimate their relative chemosensitivity. To establish a chemoresistance score for all the drugs, we selected the concentration of each drug in which the difference between patients was the largest. Based on these data, we classified patients from more resistant to more sensitive (Table 1). Each patient-derived cell line showed its own chemogram (Figure 5 and Table 1). CRCM04, CRCM12, CRCM15, CRCM16, and CRCM09 were the five most sensitive patient-derived cell lines to gemcitabine; CRCM03, CRCM16, CRCM04, CRCM12, and CRCM05 were the most sensitive to SN-38 (the active irinotecan metabolite); CRCM04, CRCM10, CRCM06, CRCM14, and CRCM13 were the most sensitive to

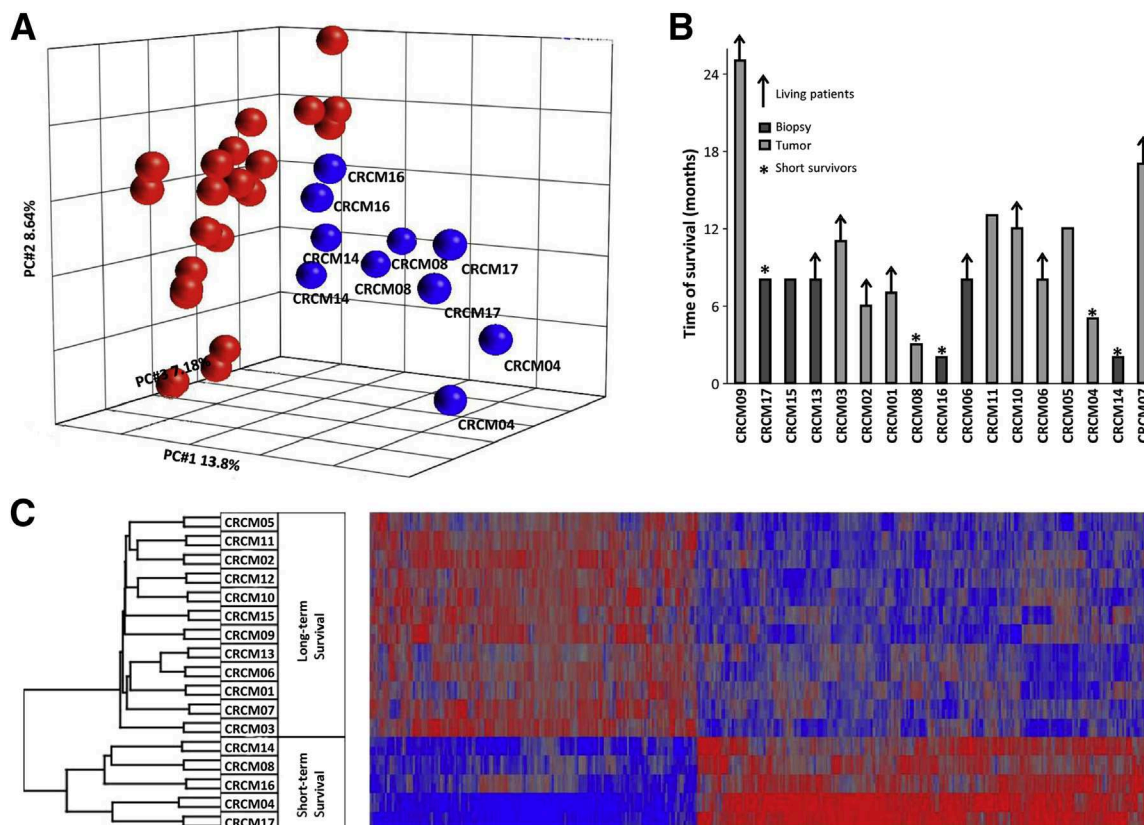


Figure 3 RNA expression analysis of pancreatic ductal adenocarcinoma. **A:** Principal component analysis obtained from the human oligonucleotide array Human Gene 2.0 Affymetrix data. Each patient is represented in duplicate because data were obtained from two independent experiments. Red plots represent long-term survival patients; blue plots, short-term survival patients. **B:** Histogram of the survival times of patients included in this study. **C:** Heat map shows the RNA expression profile of patients with long- and short-term survival.

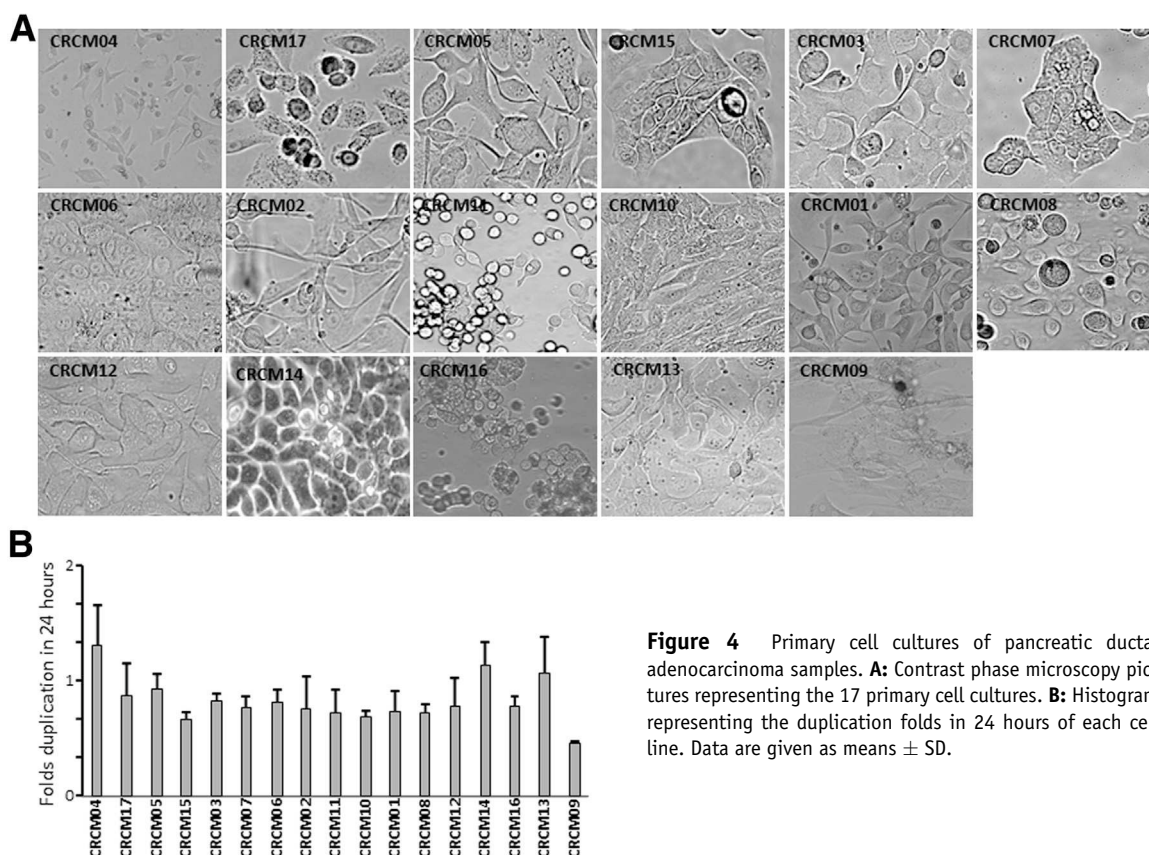


Figure 4 Primary cell cultures of pancreatic ductal adenocarcinoma samples. **A:** Contrast phase microscopy pictures representing the 17 primary cell cultures. **B:** Histogram representing the duplication folds in 24 hours of each cell line. Data are given as means $\pm$ SD.

5FU; CRCM14, CRCM04, CRCM07, CRCM16, and CRCM06 were the most sensitive to oxaliplatin; and CRCM04, CRCM08, CRCM15, CRCM14, and CRCM16 were the most sensitive to docetaxel (Table 1). We found that the five most resistant cell lines were CRCM14, CRCM13, CRCM06, CRCM17, and CRCM11 to gemcitabine; CRCM09, CRCM17,

CRCM02, CRCM10, and CRCM11 to SN-38; CRCM02, CRCM08, CRCM11, CRCM01, and CRCM12 to 5FU; CRCM08, CRCM12, CRCM01, CRCM11, and CRCM03 to oxaliplatin; and CRCM09, CRCM01, CRCM17, CRCM11, and CRCM02 to docetaxel (Table 1). Altogether, these results suggest that each PDAC-derived primary culture line has a particular profile of response. In other words, sensitivity or resistance to one drug does not predict sensitivity or resistance to another drug.

After 72 hours of incubation, some drugs were unable to kill all the cells even at very high concentrations, such as 1000 $\mu\text{mol/L}$ for gemcitabine, oxaliplatin, docetaxel, or 5FU or 100 $\mu\text{mol/L}$ for SN-38 (Figure 5). For example, 20% to 50% of cells were resistant to 1000 $\mu\text{mol/L}$ of gemcitabine; 5% to 30% remained alive when treated with 100 $\mu\text{mol/L}$ of SN-38; 10% to 70% when treated with 1000 $\mu\text{mol/L}$ 5FU; and 0% to 25% for 1000 $\mu\text{mol/L}$ oxaliplatin. A particular behavior was found when cells were treated with docetaxel; whereas doses of some drugs as low as 0.001 $\mu\text{mol/L}$ were able to kill 90% to 20%, depending on the primary culture, increasing the doses to 3.9 $\mu\text{mol/L}$ resulted in almost no change in their effect. However, almost all the cells were killed by 62.5 $\mu\text{mol/L}$. These results suggest that there is more than one cellular population in the tumor, each with different sensitivity, including complete insensitivity to drugs, which is in agreement with the intratumoral heterogeneity of PDAC. For this reason, we believe that the

Table 1 Resistance Score by Cell Line

Score	Gemcitabine	Docetaxel	5FU	Oxaliplatin	SN-38
1	CRCM04	CRCM04	CRCM04	CRCM14	CRCM03
2	CRCM12	CRCM08	CRCM10	CRCM04	CRCM16
3	CRCM15	CRCM15	CRCM06	CRCM07	CRCM04
4	CRCM16	CRCM14	CRCM14	CRCM16	CRCM12
5	CRCM09	CRCM16	CRCM13	CRCM06	CRCM05
6	CRCM10	CRCM06	CRCM07	CRCM13	CRCM06
7	CRCM08	CRCM05	CRCM05	CRCM10	CRCM15
8	CRCM03	CRCM10	CRCM09	CRCM15	CRCM13
9	CRCM05	CRCM12	CRCM03	CRCM17	CRCM07
10	CRCM07	CRCM03	CRCM15	CRCM05	CRCM14
11	CRCM01	CRCM07	CRCM17	CRCM09	CRCM01
12	CRCM02	CRCM13	CRCM16	CRCM02	CRCM08
13	CRCM11	CRCM02	CRCM12	CRCM03	CRCM11
14	CRCM17	CRCM11	CRCM01	CRCM11	CRCM10
15	CRCM06	CRCM17	CRCM11	CRCM01	CRCM02
16	CRCM13	CRCM01	CRCM08	CRCM12	CRCM17
17	CRCM14	CRCM09	CRCM02	CRCM08	CRCM09

5FU, 5-fluorouracil.

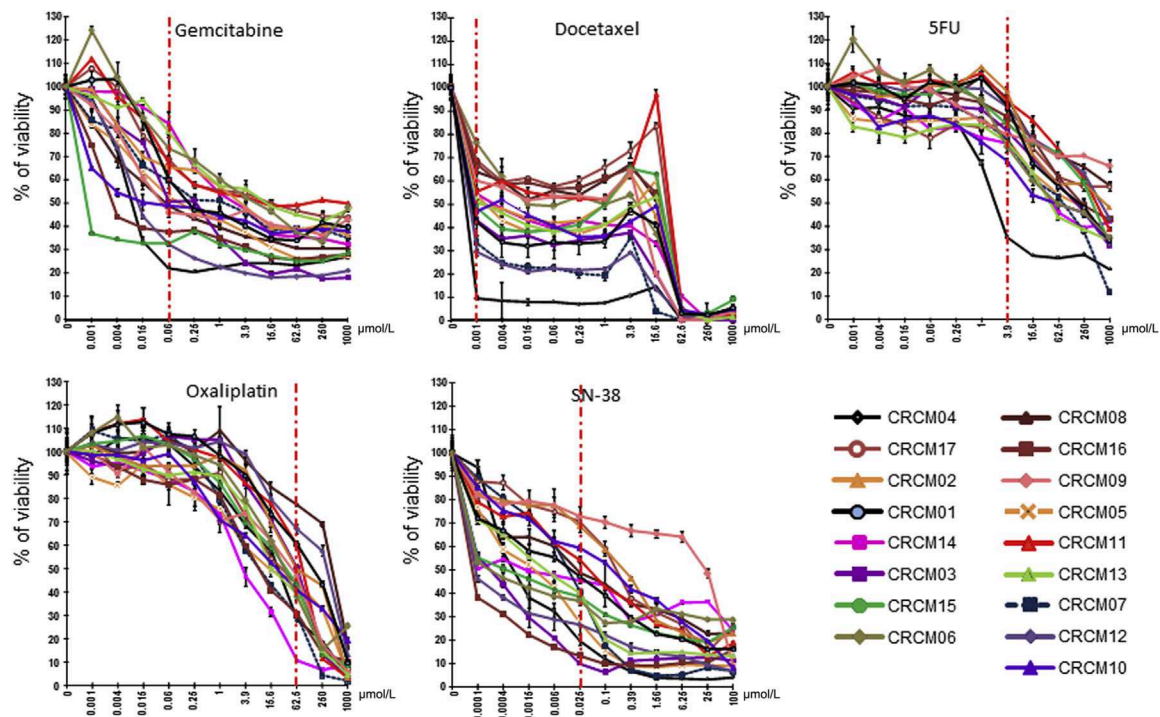


Figure 5 Chemograms. Pancreatic ductal adenocarcinoma–derived cells were treated with increasing concentrations of gemcitabine, docetaxel, 5-fluorouracil (5FU), oxaliplatin, and the active metabolite of irinotecan known as SN-38, and the survival cells were measured after 72 hours of treatment. A sensitivity profile was obtained for each drug. The point indicated with a red dotted line was used to establish the score used in Table 1.

inhibitory concentration of 50% value is not useful to characterize drug sensitivity for primary cultured cells.

Correlation between Transcriptome and Drug Resistance in PDAC

To study whether there is a correlation between drug response and transcriptome, we arbitrarily selected the four most sensitive and the four most resistant primary cultures and performed a heat map analysis of the transcriptome. Sets of genes were identified as specifically overexpressed or underexpressed in resistant and sensitive cells (Figure 6). These modulations in the expression profiles allowed us to identify 671 genes associated with gemcitabine, 1107 with oxaliplatin, 308 with 5FU, 149 with docetaxel, and only 46 with SN-38 (Figure 6A and Supplemental Tables S3–S7, respectively). Ninety-eight genes were common between oxaliplatin and 5FU, which is in agreement with their close mechanism of action. On the contrary, there were a small number of common genes associated with sensitivity and resistance to the other drugs. Few genes were observed to be common between different drugs (Figure 6B), suggesting that the phenotype of the sensitivity or resistance is specific for each drug.

Last, another important point is that genes associated with sensitivity or resistance to the drugs were not common with genes associated with short- or long-term survival (Figure 7), indicating that survival and drug sensitivity are regulated by independent mechanisms.

Discussion

We developed an efficient strategy in which PDAC tumors from 17 consecutive patients were collected, their cells maintained as primary cultures, and surgical specimens and EUS-FNA biopsy samples as breathing tumors by xenografting in immunosuppressed mice. For all the patients, clinical follow-up was conducted. Primary culture of cells allowed analysis of their relative sensitivity to several anticancer drugs *in vitro*, obtaining an individual chemo-gram. We studied the RNA expression profile using a microarray approach on breathing tumors. We used xenografts as a source of RNA rather than cultured cells because their morphologic features and microenvironment, which both have a determinant effect on RNA expression,¹³ were closer to the original human PDAC. These data were used to detect a possible correlation with the clinical outcome of each patient and with their drug responsiveness.

Methodologically, note that we were able to obtain PDAC samples from EUS-FNA and from surgically resected tumors. Biopsy samples obtained from EUS-FNA containing only some hundreds of tumor cells were able to reconstitute a tumor in mice with morphologic features very close to those of the primary tumors (Figure 1). This was systematically observed when comparing xenografts removed from mice with the histologic slide of the same resected tumor after the patient's surgery. This original strategy opens up new possibilities of clinical interest. We can imagine studying clinical outcome or the presence of a

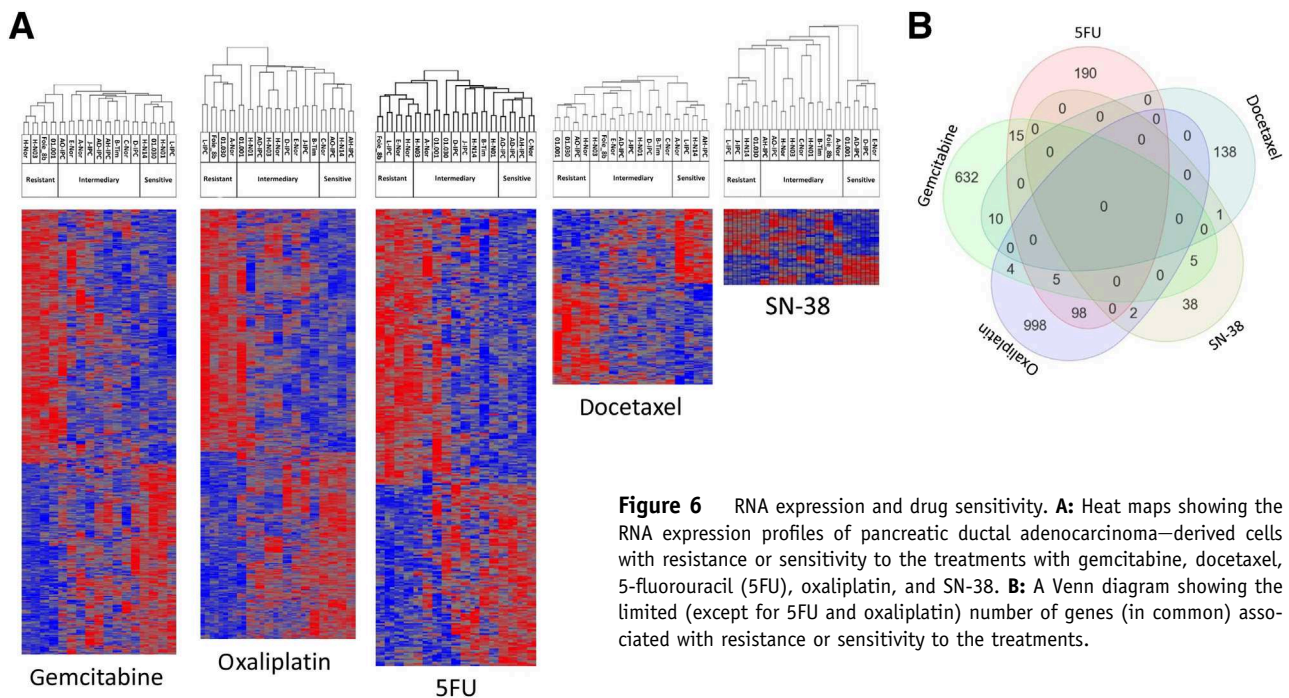


Figure 6 RNA expression and drug sensitivity. **A:** Heat maps showing the RNA expression profiles of pancreatic ductal adenocarcinoma–derived cells with resistance or sensitivity to the treatments with gemcitabine, docetaxel, 5-fluorouracil (5FU), oxaliplatin, and SN-38. **B:** A Venn diagram showing the limited (except for 5FU and oxaliplatin) number of genes (in common) associated with resistance or sensitivity to the treatments.

drug-responsive transcriptomic signature in every PDAC patient to personalize treatment. Indeed, not only could the 15% to 20% of operated patients benefit from individualized treatments but virtually all patients with a PDAC because they undergo EUS-FNA biopsy before starting chemotherapy or radiotherapy.

Analysis of gene profiling of whole PDAC tumors has largely been reported.^{14–24} However, the low reproducibility of data discouraged us from continuing in this direction. The reasons for the failure of this approach are probably associated with inappropriate strategies, inefficient tools, and the poor quality of the samples, and they can also be inherent to the pathologic disorder itself because PDAC tumors can contain 15% to 90% of stroma associated with variable areas of inflammation and necrosis. An efficient approach using laser capture of the transformed epithelial cells followed by their pangenomic expression analysis was more recently reported.²⁵ This approach demonstrated that there is a significant correlation between the expression profile and the patient outcome, including the responsiveness to some treatments. One of the limitations was that analysis was performed on only surgically resected tumors, which correspond to a minority of patients with a relatively better prognosis because of earlier detection. The other limitation was that tissue samples were obtained from formalin-fixed, paraffin-embedded tissue specimens collected between 1993 and 2006, and the quality of the obtained RNA could be deficient.²⁶ Using the EUS-FNA approach, all the patients can be included and RNA extracted from fresh tissue, providing a better quality of RNA samples for analysis.

One strength of this study is the predictive capacity of the RNA profile obtained to separate at least two groups of

patients: those with short-term survival (≤ 8 months) and those with long-term survival (> 8 months). Analysis of clinical data from short- and long-term survival patients showed that the first group had poorly differentiated tumors, whereas long-term survival patients presented with middle- or well-differentiated tumors. As expected, this observation matched with the morphologic features observed in xenografted tumors (Figure 1). Therefore, these data are not surprising because tumors with low-grade differentiation are expected to be associated with short-term survival compared with high- or middle-grade differentiation.^{27–30} This correlation between tumor differentiation and prognosis was already described for pancreatic neuroendocrine tumors,³¹ and its pertinence in PDAC was recently documented by Wasif et al,³² who described a correlation between tumor differentiation and patient survival time as well as a value to predict the response to treatment. In fact, tumor differentiation or grade is increasingly used as an independent prognostic factor of survival, it seems with as much impact as prognostic tumor size or lymph node metastatic invasion.³³ Although the grade of differentiation can be estimated by the pathologist after a pancreatectomy, this is possible in only approximately 15% of patients. However, using a set of molecular markers identified in this study, differentiation could be estimated using only a few cells obtained by EUS-FNA. In fact, based on the present results, it seems evident that using the expression of some of these molecular indicators on small PDAC samples as obtained by EUS-FNA would help in predicting the behavior of the PDAC and, therefore, conditioning the therapeutic strategy. Although this application sounds very exciting, it needs to be validated in an independent cohort of patients.

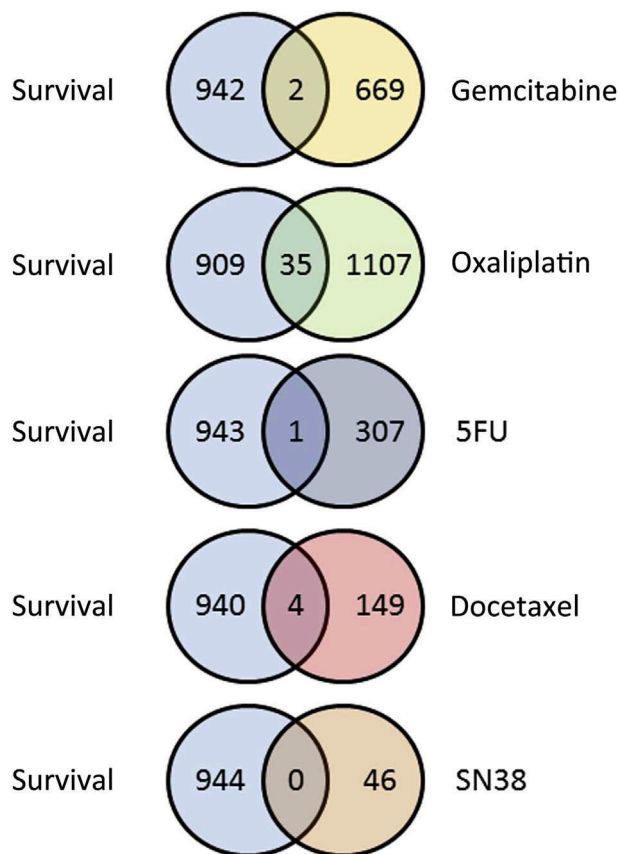


Figure 7 A Venn diagram showing the limited number of genes (in common) associated with resistance or sensitivity to the treatments with those discriminating patients with long- and short-term survival. 5FU, 5-fluorouracil.

Yachida et al³⁴ analyzed the effect of the more frequent somatic genetic alterations—inactivation of *CDKN2A*, *SMAD4*, and *TP53* and mutation of *Kras*—on the outcome of patients with a PDAC and found a direct association between accumulation of these genetic abnormalities and short survival. Another important fact to consider concerns data recently reported by Garrido-Laguna et al,³⁵ who suggest that xenografts from PDAC tumors grow preferentially when patients have a poor prognosis, indicating a bias for the xenograft approach. From their results, the best-growing xenografts are from patients with inactivation of *SMAD4*, resistance to gemcitabine, and a gene expression profile enriched in the Notch pathway. This observation seems to make sense but needs to be confirmed by other laboratories. In the present case, of the xenografts studied, only a few showed *SMAD4* inactivation (data not shown), sensitivity to gemcitabine was very variable (Figure 5), and genes involved in the Notch pathway showed no particular enrichment (data not shown). However, consistently, aldehyde dehydrogenase activity in xenografts was not determinant in this study because we found 0% to 34% positive cells (data not shown).

Another clinically relevant observation concerns the expression profile associated with resistance to some anticancer drugs. Indeed, we observed that 671 genes for gemcitabine,

1107 for oxaliplatin, 308 for 5FU, 149 for docetaxel, and 46 for SN-38 were able to discriminate between tumors sensitive and resistant to each drug. The chemogram we developed includes the most efficient drugs used for treating PDAC patients. Whereas the gene profile associated with gemcitabine, oxaliplatin, and 5FU sensitivity included numerous genes, the number of genes associated with docetaxel and SN-38 was relatively small, suggesting that in the first case the transcriptomic phenotype could be determinant, whereas in the second case this phenotype could be less important. Only a limited number of genes were common between the different drugs, except for oxaliplatin and 5FU (Figure 6B), which is not surprising given that both drugs kill the cell by a related mechanism.^{36,37} Sensitivity to the treatments was patient specific and was not associated with the differentiation degree of the tumor or with the doubling time shown by the primary culture of cancer cells (Figure 4B), which is contrary to expectations, indicating complex molecular mechanisms regulating chemoresistance. Another important observation was that in some cases drugs were unable to kill all the cells, even at concentration as high as 1000 $\mu\text{mol/L}$ (Figure 5), making it impossible to establish their inhibitory concentration of 50% value. This observation can be explained by the fact that primary cultures are representative of the different cell populations present in the tumor, as heterogeneous as PDAC is known to be.³⁸ This could be clinically relevant because the chemogram may detect the percentage of sensitive and resistant cells for a drug and, therefore, may become a helpful tool for the oncologist selecting the second line of treatment for a patient. Other authors have reported a differential sensitivity to anticancer drugs in low-passage pancreatic cancer–derived cells, but no correlation with their RNA profile was reported.^{39,40} However, these authors reported that inactivation of *SMAD4* and *CDKN2A* was associated with resistance to cisplatin and irinotecan and to gemcitabine and mitomycin C, respectively.⁴⁰ Similarly, as for defining whether a PDAC patient will be a short or long survivor, as discussed in *Materials and Methods*, it would be clinically relevant to determine the expression pattern associated with the sensitivity of the anticancer drugs. In fact, determining the expression of a small number of genes on samples obtained by EUS-FNA could be the first step toward a personalized oncology for PDAC.

Note that the set of genes selected as markers to separate short-term from long-term survival patients were different from genes selected to define sensitivity to the drug (Figure 7). This observation suggests, as expected, that mechanisms of drug resistance are independent of mechanisms defining spontaneous evolution of the disease. We presented data obtained from a relatively small number of samples, but, to our surprise, differences were frequently highly significant, suggesting that this approach is robust. Therefore, we can expect that increasing the number of patients will increase the sensitivity and specificity of data, with a more limited number of genes to be retained to constitute the signature able to determine drug sensitivity and patient outcome.

In conclusion, we showed that transcriptomic analysis of PDAC xenografts can discriminate short- and long-term survivors and can predict sensitivity to a set of anticancer drugs.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpathol.2014.11.029>.

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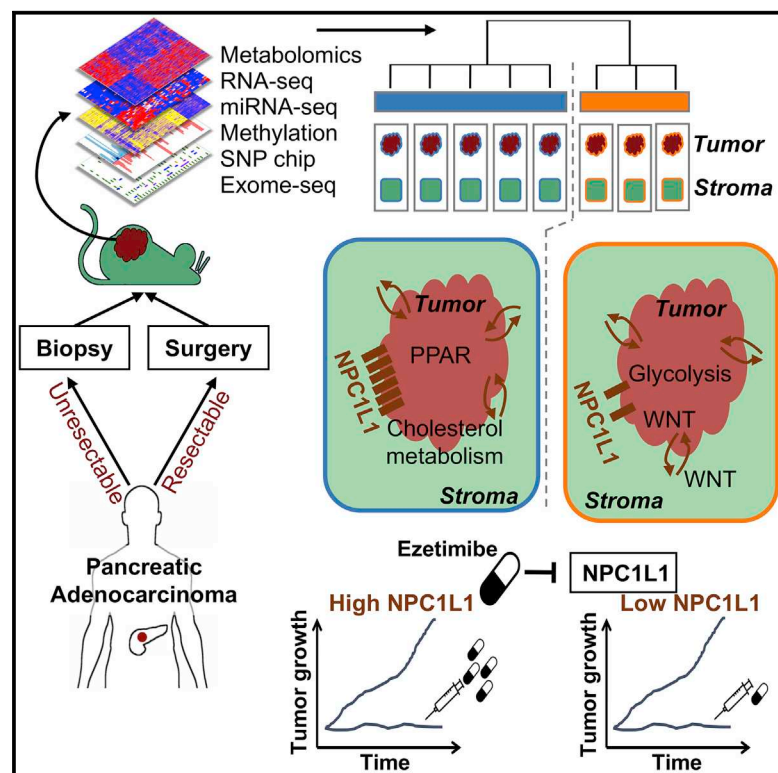
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Cell Reports

Pancreatic Adenocarcinoma Therapeutic Targets Revealed by Tumor-Stroma Cross-Talk Analyses in Patient-Derived Xenografts

Graphical Abstract



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In Brief

Nicolle et al. present a genomic analysis of pancreatic cancer xenografts showing that tumor subtypes are defined by specific epigenetic, transcriptional, and stromal landscapes. They reveal potential therapeutic targets through analysis of signaling cross-talk between tumor and stromal cells.

Highlights

- Patient xenografts are relevant models of pancreatic cancers and of their stroma
- Xenografts allow the genomic analysis of unresectable pancreatic cancers
- The epigenome, transcriptome, and stroma of pancreatic cancer define two subtypes
- Pancreatic cancer is sensitive to inhibition of *NPC1L1* by Ezetimibe



Pancreatic Adenocarcinoma Therapeutic Targets Revealed by Tumor-Stroma Cross-Talk Analyses in Patient-Derived Xenografts

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SUMMARY

Preclinical models based on patient-derived xenografts have remarkable specificity in distinguishing transformed human tumor cells from non-transformed murine stromal cells computationally. We obtained 29 pancreatic ductal adenocarcinoma (PDAC) xenografts from either resectable or non-resectable patients (surgery and endoscopic ultrasound-guided fine-needle aspirate, respectively). Extensive multiomic profiling revealed two subtypes with distinct clinical outcomes. These subtypes uncovered specific alterations in DNA methylation and transcription as well as in signaling pathways involved in tumor-stromal cross-talk. The analysis of these pathways indicates therapeutic opportunities for targeting both compartments and their interactions. In particular, we show that inhibiting NPC1L1 with Ezetimibe, a clinically available drug, might be an efficient approach for treating pancreatic cancers. These findings uncover the complex and diverse interplay between PDAC tumors and the stroma and demonstrate the pivotal role of xenografts for drug discovery and relevance to PDAC.

INTRODUCTION

Stratifying tumors using genome-wide molecular profiles has proven to be valuable for predicting therapeutic responses and

clinical outcome in many neoplastic diseases. While pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths, few integrative genomic, epigenomic, and transcriptomic studies have been conducted (Bailey et al., 2016; Cancer Genome Atlas Research Network [TCGA], 2017). This is mainly due to difficulty in obtaining an appropriate series of PDAC tumor samples. A major obstacle is the requirement for surgical samples resulting in the exclusion of the 85% non-resectable patients. Another critical issue is the high proportion of non-transformed stromal cells infiltrating the tumor, which greatly hinders the analysis of carcinogenic-specific processes. Despite these difficulties, the diversity of PDAC has been investigated recently using genome-wide approaches on surgical samples. A canonical study combining elegant laser capture microdissection followed by transcriptomic analysis revealed three main groups of patients: classical, quasi-mesenchymal, and exocrine-like (Collisson et al., 2011). The defined subtypes were suggested to differ in their clinical outcome and therapeutic responses. Another interesting study designed for tumor stratification and drug sensitivity reproduced this classification on a small number of samples (Noll et al., 2016). Moffitt and colleagues (Moffitt et al., 2015) involved a sophisticated computational approach using transcriptomic data and isolated cancerous, stromal, and normal tissue gene expression profiles. Analysis of estimated cancer-specific profiles identified two main tumor subtypes, including a basal-like subtype with a poor clinical outcome that bears great similarity to the basal-like tumors described in bladder and breast cancers. This study suggested that the exocrine-like subtype is attributable to the presence of normal non-transformed pancreatic

endocrine/exocrine epithelium. More recently, the Australian pancreatic cancer ICGC (International Cancer Genome Consortium) project defined four subtypes of PDAC based on the transcriptomic analysis of 96 selected primary tumors (Bailey et al., 2016), which they named squamous, pancreatic progenitor, immunogenic, and aberrantly differentiated endocrine exocrine (ADEX). Overall, solely based on gene expression, these studies recurrently found one specific subtype associated with a poor prognosis and a loss of differentiation that was either termed quasi-mesenchymal, basal-like, or squamous. However, no consensus arose from the subdivision of the well-differentiated PDAC tumors, particularly as none of the non-basal subtypes that were proposed are distinguishable based on their clinical outcome. In addition to the lack of non-resectable tumors, only one study proposed a model of the function and diversity of PDAC stroma (Bailey et al., 2016; Moffitt et al., 2015). Indeed, while the extensive desmoplasia present in PDAC tumors has raised major interrogations of its clinical impact and, more generally, on its function in the carcinogenic process (Carapuça et al., 2016; Rhim et al., 2014; Sherman et al., 2014; Tape et al., 2016), large-scale studies of the pancreatic tumor microenvironment are lacking.

Patient-derived xenografts (PDXs) are progressively appearing as a prime approach for much-needed preclinical studies and, in particular, to characterize drug efficacy by simulating phase II clinical trials (Gao et al., 2015; Townsend et al., 2016). PDXs have major advantages over cell lines as models of primary cancers. For instance, tumor cells preserve their complex 3D organization by being embedded in an active microenvironment with an elaborate and dynamic cellular composition. Importantly, xenografts of human primary PDAC enable “*ex patient*,” but *in vivo*, studies and through consecutive passages allow characterization of the tumor even after patient mortality. Although there are limitations, particularly related to the host environment, PDX is probably the closest currently available model to the human disease. Several studies in bladder (Pan et al., 2015), colorectal cancer, and lymphoma (Song et al., 2016) have shown the similarities between primary tumors and PDX, with a particular emphasis on genomic alterations. Importantly, a study on PDAC and hepatocellular carcinoma PDX suggested that the main differences in gene expression between primary tumors and xenografts are due to the difficulty of profiling the murine stroma using microarrays (Martinez-Garcia et al., 2014), as they measure human gene expression. With the advent of sequencing-based transcriptomic profiling, PDX offers an ideal setting to distinguish and study the interactions between the tumor and stromal cells. Indeed, sequencing profiles of a mix of human grafted cancerous and infiltrating mouse stromal cells can be analyzed separately *in silico* by unambiguously assigning each sequence to the human or mouse genome (Bradford et al., 2013). However, although PDX models are increasingly used in preclinical studies, they are insufficiently characterized, as few studies have addressed the question of their relevance as a model of the diversity of human disease (Witkiewicz et al., 2016). Finally, we expect that the integrated analysis of PDX multiomics profiles will reveal therapeutic targets for this disease.

RESULTS

PDX Establishment and Validation of the Model

Following suspicion of PDAC, the endoscopic ultrasound-guided fine-needle aspirates of non-resectable tumors or surgical samples of resectable tumors were used to generate subcutaneous xenografts in immunocompromised mice. Histological comparison of resected primary tumors with their correspondent PDX demonstrated extensive similarities (Figure S1A). Early passage of 29 patient-derived xenografts (30 xenograft samples in all) were used to generate genomic, epigenomic, and transcriptomic profiles (Figure 1A). In order to accurately analyze bulk xenograft samples, we developed a methodology that we termed SMAP (simultaneous mapping for patient-derived xenograft), which uses both the human and mouse genomes to distinguish reads from the tumor and stromal compartment, respectively (see Supplemental Information). The separation of species-specific RNA reads resulted in two transcriptomic profiles for each sample: one tumor profile based on human RNA sequences from the grafted tumor cells and one stromal profile from the mouse RNA sequences.

To validate PDX as a model of the primary tumor, the transcriptomes were compared to the virtually microdissected expression signatures that were recently proposed as associated with the tumor or stromal cells of primary human PDAC (Moffitt et al., 2015). The immune signature and the two stromal signatures (normal and activated) were predominantly expressed by the murine stromal cells, while the two cancer signatures (basal-like and classical) were expressed by the human tumor cells (Figures 1B and S1B). This clear distinction of the grafted human tumor cells and the recruited murine stromal cells provides the means to identify potential tumor-stroma interactions from the analysis of a bulk xenograft sample. The combined analysis of both murine and human transcriptomes highlighted several well-described tumor-stroma interactions, such as the activation of MET and insulin growth factor (IGF)1R in tumor cells by the stromal-expressed HGF and IGF, respectively (Figures 1C and S1C; Table S1). Interestingly, 9 out of 17 stromal genes shown in Figure 1C are part of the activated stromal signature previously defined in human primary tumors (Moffitt et al., 2015). Overall, these results illustrate the relevance of the PDX model, in particular, in the establishment of a dialog between the grafted cells and their host microenvironment.

Mirrored Tumor-Stroma Classification

In order to uncover the tumor diversity of PDAC using PDX, unsupervised analyses were carried out independently on each of the tumor-specific genome-wide molecular profiles: protein coding mRNA, long non-coding RNA, microRNA, and non-island and island CpG methylation. The resulting classifications consistently characterized two subtypes defined by the nearly identical sets of samples (Figures 2A and S2A–S2F; Table S2). This marked convergence was summarized in a consensus multiomics classification composed of two subtypes, basal (orange) and classical (blue), as well as two samples with discrepancies between their single-omics classification that will be referred to as outliers (gray) (Figure 2A). Although non-island methylation was consistent with the other single-omics, the island

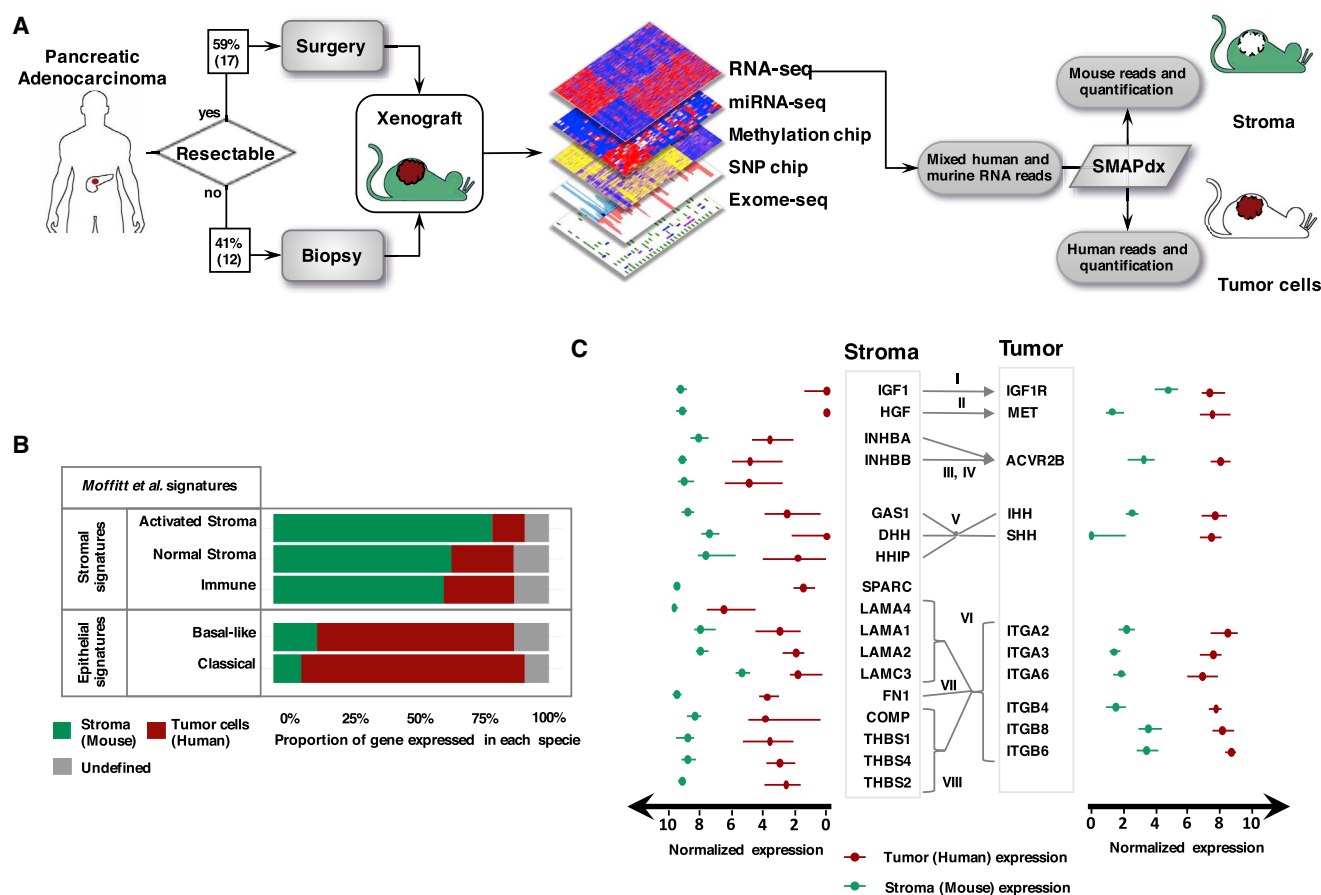


Figure 1. Patient-Derived Pancreatic Adenocarcinoma Xenograft Generation and Characterization

(A) Experimental design for systematic PDX generation followed by molecular tumor and stromal cell characterization. Numbers of biopsy and surgical samples are indicated.

(B) Expression of human primary PDAC stromal and tumor signatures in the mouse stroma or human tumor cells. Lateral stacked bars correspond to the number of genes in each signature that is specifically expressed in PDX by mouse cells (green), human cells (red), or neither (gray).

(C) Tumor (red) and stromal (green) expression of genes in pathways known to be involved in tumor/stroma cross-talk. Values shown are median expressions, and error bars indicate first and third quartiles. References: I (Bergmann et al., 1995), II (Qian et al., 2003), III (Lonardo et al., 2011), IV (Ohnishi et al., 2003), V (Tian et al., 2009), VI (Whatcott et al., 2013), VII (Topalovski and Brekken, 2016), and VIII (Farrow et al., 2009).

methylation classification revealed a subgroup of CIMP (CpG island methylator phenotype) tumors within the classical subtype (Figure 2B). Virtually, no genes were found to be significantly differentially expressed in association to the CIMP in this cohort (Figures S2G–S2I; Table S2). Moreover, the genes that were associated with the CIMP hypermethylated CpG were more often found to be underexpressed in all classical samples (64 of the 184 hypermethylated genes found significantly underexpressed in classical samples as compared to basal samples) than specifically underexpressed in CIMP samples (neither of the 2 genes were found to be underexpressed in CIMP samples). These results suggest that the CIMP is only one of the mechanism-silencing genes in classical samples, while the others are yet to be discovered.

Global genomic properties such as chromosomal instability index (CIN) or mutation rate showed no specific association with the classification (Figure 2B). Unsurprisingly, genomic alterations that are commonly found in PDAC (*KRAS* mutations,

SMAD4 losses, *CDKN2A* inactivation, *myc* amplification, etc.), were also widespread in PDX (Figure S2J). However, none of these alterations and none of any of the genomic alteration events, including potential gene fusions (Table S2), identified in this work were associated with the basal or classical subtype. As previously observed (Bailey et al., 2016), *TP53* showed a slightly higher mutation rate in the basal subtype, although with virtually no discriminative power. Besides the absence of subtype-specific single alterations, the unsupervised classification of copy number aberrations also showed no association (Figure S2K). This lack of genetic support for the two PDAC subtypes has been suggested in a larger series of patients using whole-genome sequencing (Bailey et al., 2016). However, the remarkable parallel between the transcriptome and methylome suggests that the main phenotypes in PDAC are epigenetically rather than genetically established.

Clinical and histopathological characterizations revealed that patients of the basal subtype were more often non-resectable,

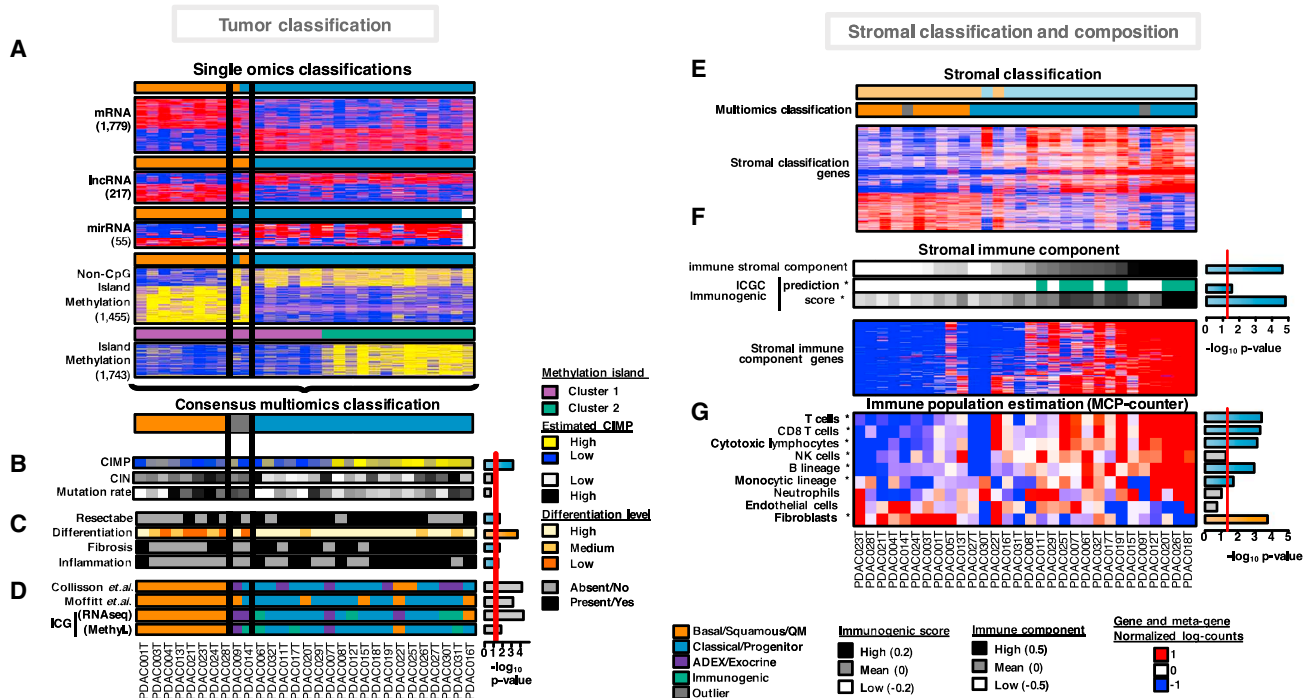


Figure 2. Tumor and Stroma Classifications

(A) Single-omics classifications and associated differential feature heatmaps used to define a consensus multiomics classification. Numbers in parentheses correspond to the number of differential features.

(B) Global epigenetic and genetic characteristics estimated from genome-wide assays. CIN, chromosomal instability index; CIMP, CpG island methylator phenotype.

(C) Clinical and histopathological characteristics.

(D) Primary human PDAC classification systems applied to the PDX tumor cell transcriptomes or methylation data (Methyl.). Lateral bars correspond to $-\log_{10}$ p value of Fisher's exact test of the association with the consensus multiomics classification (excluding outliers). Bars are colored when associated to a specific subtype (orange for basal; blue for classical) and significant ($p \leq 0.05$, indicated by red line).

(E) Consensus clustering applied to the stromal gene expression, generated from mouse RNA-seq reads. Heatmap represents expression values of all differentially expressed stromal genes between the two stromal subtypes.

(F) Immune stromal component sample projection and its contributing genes associated to the ICGC immunogenic subtype (prediction and score) applied to the stromal transcriptomes.

(G) Estimated level of infiltration of seven immune and two other stromal populations as computed by MCPcounter. Lateral bars correspond to $-\log_{10}$ p value of the association with the consensus multiomics classification (excluding outliers; Fisher's exact test or ANOVA). Bars are colored when associated to a specific subtype (orange for basal; blue for classical) and significant ($p \leq 0.05$). * $p \leq 0.05$, significant association with the stromal immune component (Pearson's correlation or ANOVA). All classifications and components analysis shown are based on unsupervised analysis of the associated datasets. Heatmaps show expression/methylation values of the genes/CpG most associated with the unsupervised classification or component. All heatmaps are shown with the same samples in the same order.

had a marginally lower median survival, and presented a less differentiated tumor (Figures 2C and S2L), altogether indicating a more aggressive phenotype. Conversely, classical tumors were more frequently resectable, presented a higher level of differentiation, and were often associated with fibrosis and inflammation. In order to compare these PDX-based subtypes with currently available human PDAC classifications, methylome and gene expression classifiers were constructed from three different public datasets (Collisson et al., 2011), (Moffitt et al., 2015), ICGC methylation (Nones et al., 2014) and RNA sequencing (RNA-seq) (Bailey et al., 2016). All published PDAC classification systems were in agreement with the proposed multiomics classification, thereby illustrating the reliability of PDX to model PDAC diversity (Figures 2D and S3A–S3D). Altogether, our analysis on cancer cells using either PDX or

human PDAC classifications reveals the presence of two clinically relevant types of PDAC.

Unsupervised analysis of the stromal transcriptomes reconstructed from the murine RNA sequences also revealed two types of stroma with a strong agreement with tumor subtypes (Figure 2E). In order to evaluate this remarkable echo in the diversity of the tumor cells and of their stroma, the tumor and stroma PDAC PDX subtypes were predicted in four public datasets of human primary PDAC. To do so, we derived a tumor and a stromal gene signature predictive of PDAC subtypes based on tumor and stromal expression, respectively. A minor overlap was found between the genes of these signatures, a majority of which encoded cell communication proteins or extra-cellular components (e.g., semaphorins, cytokines; Table S2). The stromal and tumor subtype prediction made by these gene

signatures significantly matched in all four primary human tumor datasets, which confirms the mirrored tumor-stromal classification observed in xenografts (Figure S3E).

Interestingly, two recent studies on bulk primary human pancreatic tumors proposed a classification that was suggested to be linked to the stromal composition. The first, by Moffitt et al., described an activated stroma that was linked to a poor outcome (Moffitt et al., 2015) and recently associated with the basal subtype (Bailey et al., 2016). The second was the immunogenic group, which was suggested to be driven by a strong infiltration of immune cells. In order to further characterize the stroma of PDX, a blind source separation algorithm, independent component analysis, was used to extract two components from the stromal transcriptomes (Table S2). The first component was weakly associated with the basal subtype and mostly composed of genes that define the proposed activated stroma of primary PDAC (Moffitt et al., 2015) (Figure S3F). The genes contributing to this component observed in the stroma of xenograft suggest a gradient in stromal activation. Similar to observations in primary human PDAC, the xenografts with a strong activation of their stroma have a marginal tendency to be associated with patients with lower survival (Figure S3G). The second component was strongly associated with the classical subtype as well as with the immunogenic class predictor applied to the stroma (Figure 2F). The immune aspect of this component was supported by its correlation with the infiltration of several populations of immune cells estimated using MCPcounter (Becht et al., 2016) (Figure 2G). Remarkably, these results show that, although the mouse hosts are immunodeprived, PDX tumor models are able to reproduce an immune-related phenotype observed in human primary tumors.

Basal Subtype Characterization

The precise sequence-based dissociation of tumor and stroma transcriptomic profiles offers the possibility to rigorously analyze the carcinogenic processes of tumor cells in a subtype-specific approach (Table S3). The basal subtype showed significant deregulations in oncogenic-related pathways and, in particular, a strong overexpression of cell-cycle genes (Figure 3A). The upregulation of the glycolysis in basal tumor cells is in line with a recent description of a group of glycolytic PDAC cell lines associated with a characteristic mesenchymal phenotype (Daemen et al., 2015). Several pathways were also associated with a differential methylated pattern as well as miRNA-based deregulations. This supports the hypothesis that PDAC diversity and the specificities in carcinogenic development of each subtype are epigenetically rather than genetically driven.

In addition to the description of basal tumor cell pathways, the differential analysis of basal stromal pathways was also carried out. The genes involved in focal adhesion and axon guidance pathways, which imply complex interactions with the surrounding tissues, were overexpressed by the tumor and stromal compartments. Along with the enrichment of basal PDX in fibroblasts (Figure 2G), this result suggests a basal-specific communication between tumor cells and adjacent fibroblasts, which is in agreement with recent observations in PDAC of the role of cancer-associated fibroblasts in axon guidance signaling (Secq et al.,

2015). In order to validate the transcriptional activation of these pathways in human primary PDAC, an identical pathway analysis was performed on three independent datasets (Figure 3A). The results consistently showed that human basal PDAC activated all of the pathways highlighted in basal PDX. Moreover, the comparison between basal cancer and normal pancreatic transcriptomes showed that these pathway upregulations were cancer specific. Figure 3B schematically illustrates the means by which transcriptional and epigenetic alterations stimulate the highly deregulated WNT pathway. Wnt signaling is potentially activated by tumor cells in an autocrine manner through the upregulation of several WNT ligands, as well as in a paracrine manner by the stromal upregulation of distinct WNT ligands. Finally, the analysis of the methylation patterns suggests a sustained activation of the entire pathway as shown by aberrant methylation of effectors at nearly every step of the signaling cascade as well as of inhibitors of the pathway (Figure S4A).

Classical Subtype Characterization

The analysis of transcriptionally upregulated genes in classical PDX revealed the activation of many pathways (Figure 4A). The upregulation of all of the highlighted pathways was verified in an equivalent differential analysis on human primary PDAC. Importantly, several of these were also found to be active in normal pancreatic samples, suggesting an aberrant differentiated phenotype. Interestingly, a majority of classical-specific pathways relate to either pancreatic digestive function (fat and protein digestion, pancreatic and bile secretion), metabolite transport (fat and protein absorption, small molecule transport), or metabolic pathways (glucose, fructose, mannose, arginine, proline, linoleic, and arachidonic acid metabolism). These results suggest that Classical tumor cells acquire cellular functions usually imputed to other gastrointestinal tissues (e.g. enterocytes), in addition to retaining some level of pancreatic digestive function. This is in line with the previous description of the Classical subtype as Progenitor, although we speculate it may acquire some characteristics of a more general gastrointestinal progenitor. Unlike the previously proposed exocrine-like or ADEX PDAC subtypes, we observe here that the Classical subtype maintains a lower level of a more general digestive activity, including product breakdown and uptake that could be used to fuel high metabolic needs. Altogether, we describe the Classical subtype as closer to normal pancreatic tissue than the Basal subtype yet, we do not identify any acinar-like or any differentiated pancreatic subtypes which would only harbor functions and markers of the exocrine or endocrine pancreas.

In order to further characterize the molecular phenotypes of these PDXs, extensive metabolomics profiles, including lipidomics, were generated. Differential analysis of metabolite quantifications supported this hypothesis by demonstrating a general increase in REDOX-related metabolites (Figure S4B; Table S4), previously associated with well-differentiated PDAC cell lines (Daemen et al., 2015). The potential digestive activity was particularly demonstrated by the decline in triacylglycerols associated with an increased lipase expression and an increased level of fatty acids (Figure 4B). In addition, classical tumors also displayed an increase in several glycerophospholipids

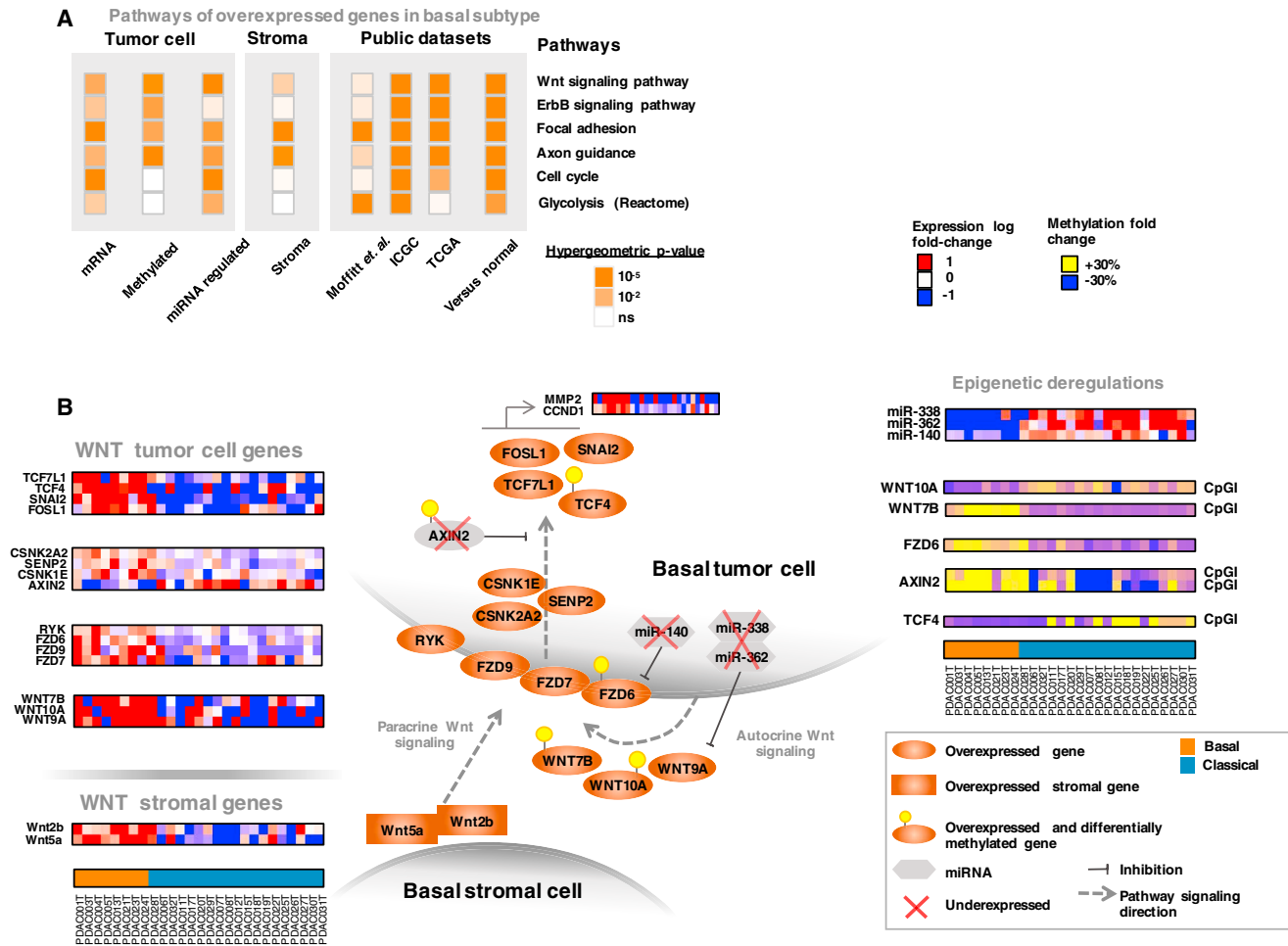


Figure 3. Basal Epigenetically Deregulated Pathways

(A) Basal pathway enrichment of: overexpressed genes (mRNA), overexpressed and differentially methylated genes (Methylated), overexpressed genes that are targets of underexpressed microRNA (miRNA regulated), genes overexpressed in the stroma of basal tumors (Stroma), overexpressed genes in the basal tumors of the *Moffitt et al. (2015)* dataset (Moffitt et al.), the ICGC dataset (ICGC), and the TCGA dataset (TCGA), and genes overexpressed in basal tumors compared to normal pancreatic samples (Versus normal). Pathway definition originates from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database unless specified otherwise.

(B) Schematic illustration of the tumor and stromal deregulation of the WNT signaling pathway. Ellipses represent genes and hexagons miRNA found to be overexpressed (orange) or underexpressed (gray and crossed) in basal PDX tumors. Yellow circles indicate methylation-associated deregulation of expression. Rectangles represent genes overexpressed in basal PDX stroma. Blue-red heatmaps represent expression values of genes and miRNA. Blue-yellow heatmaps represent methylations values of CpG associated with genes. The CpGI annotation indicates that represented CpG methylation level originates from a CpG island. All heatmaps are shown with the same samples in the same order.

(Figure S4C), altogether indicating a broad deregulation of the lipid metabolism, as suggested in a previous metabolomics study on PDAC cell lines (Daemen et al., 2015).

Figure 5 shows a partial view of the range of small molecule transporters overexpressed in classical samples, which include the upregulation of the glucose (*SLC2A2*) and glutamine (*SLC1A7*) transporters, which are strongly hypomethylated in classical samples (Figure S4D). Glutamine is a necessary nitrogen donor for the initial steps of nucleotide biosynthesis. Interestingly, the upregulation and epigenetic deregulation of the glutamine transporter in classical tumors is associated to a strong increase in two nucleotides, inosine monophosphate

(IMP) and uridine monophosphate (UMP) (Figure S4E). Cholesterol transporters were also significantly upregulated; in particular, the overexpression of the intestinal cholesterol uptake regulator *NPC1L1* was associated to extensive hypomethylation (Figure 5). Aberrant cholesterol uptake has been recently implicated in the proliferation and survival of pancreatic cancer cells (Guillaumond et al., 2015). Metabolomics profiling revealed a significantly higher level of cholesteryl ester in classical PDX (Figure 5), supporting an increased absorption activity. Moreover, the analysis of the stroma of classical tumors highlighted the upregulation of genes involved in lipid metabolism and cholesterol synthesis, as well as the master regulators of lipid

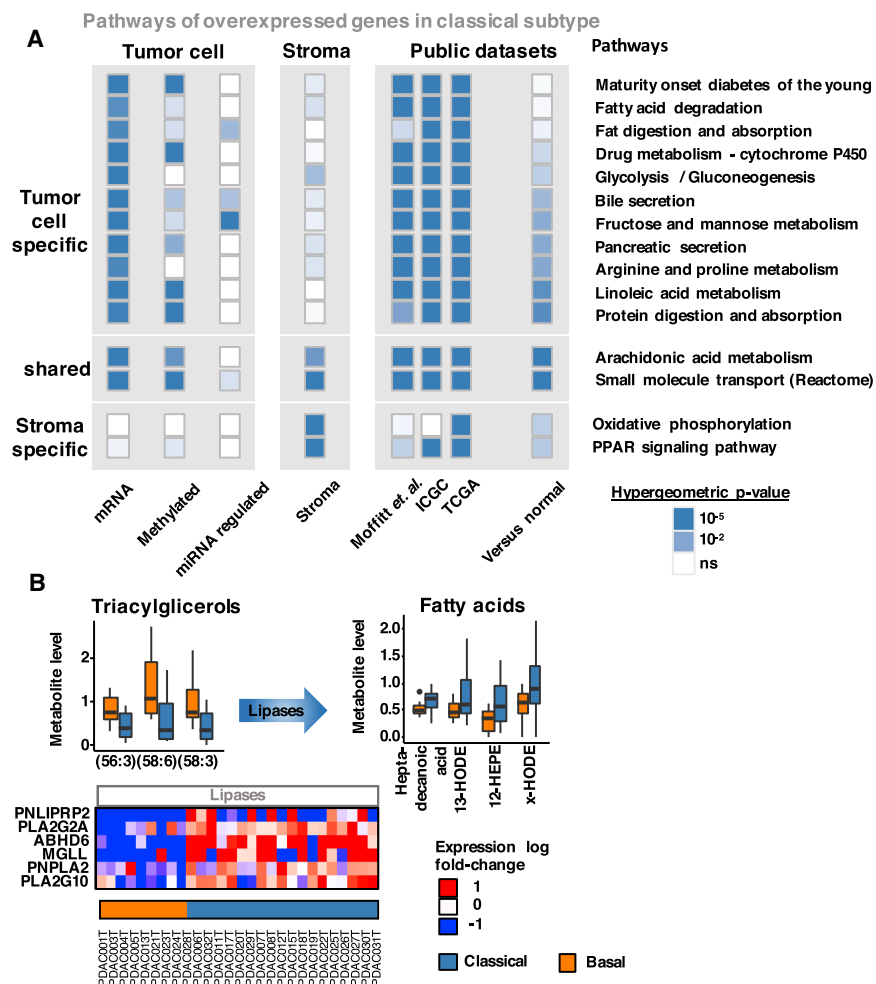


Figure 4. Classical Signaling and Metabolic Pathways

(A) Classical pathway enrichment of: overexpressed genes (mRNA), overexpressed and differentially methylated genes (Methylated) overexpressed genes that are targets of underexpressed micro-RNA (miRNA regulated), genes overexpressed in the stroma of classical tumors (Stroma), overexpressed genes in the classical tumors of the Moffitt et al. (2015) dataset (Moffitt et al.), the ICGC dataset (ICGC) and the TCGA dataset (TCGA), and genes overexpressed in Classical tumors compared to normal pancreatic samples (versus normal). Pathways are ordered by their tumor specificity compared to normal pancreatic samples and to the stroma. Pathway definition originates from the KEGG database, unless specified otherwise.

(B) Pancreatic lipase activity. Boxplots show the levels of the putative metabolic substrates (triacylglycerols, left) and products (fatty acids, right) of lipases in basal and classical samples. Expression of lipases is represented as a heatmap.

and cholesterol homeostasis, namely, *PPARG* and *NR1H3* (LXR α). Altogether, these results suggest an active cross-talk involving the stromal synthesis of cholesterol associated with aberrant nutrient uptake by classical tumor cells.

In order to validate the predicted compartment-specific expression of genes of interest, immunohistochemical staining was performed. As a control, *MUC17*, which was expressed by classical tumor cells displayed no protein expression in basal tumors, as expected (Figures S5A and S5B). Immunohistochemical staining of *PPARG* and its target *FABP5* confirmed transcriptomic data by validating their expression in classical stromal cells (Figures S5C and S5D).

Evidence of Potential Therapeutic Targets

Despite the absence of any specific druggable genetic alterations, the integrative multiomics analysis uncovered epigenetically deregulated pathways in PDAC subtypes with high potential druggability (e.g., *WNT*, *EGFR*, *PPARG*, small molecule transporters). In particular, our integrated analysis examined the stroma as well as the epigenetic deregulation of cholesterol metabolism and uptake. Moreover, cholesterol metabolism was recently associated with pancreatic cancer in cellular and

epidemiological studies (Chen et al., 2015; Guillaumond et al., 2015). As a proof of concept for defining therapeutic targets from the integrated multiomics analysis of PDX, we selected the highly epigenetically deregulated *NPC1L1*, for which an efficient inhibitor is clinically available: Ezetimibe. DNA demethylation using 5-Aza-2'-deoxycytidine confirmed the epigenetic regulation of *NPC1L1* (Figure S6A). Immunohistochemical staining confirmed the protein expression of *NPC1L1* and its correlation with mRNA levels, overall showing higher levels in classical tumors (Figures 6A and S6B).

Sensitivity analysis of Ezetimibe on the survival of 4 basal and 3 classical PDX-derived cell cultures expressing different levels of *NPC1L1* revealed that all PDAC cells are sensitive to the inhibitor, while the treatment had little to no effect on fibroblasts (Figures 6B and S6C). However, as Ezetimibe functions as a competitor of cholesterol, cells expressing lower levels of *NPC1L1* (i.e., Basal) were highly sensitive, while cells with high levels of *NPC1L1* (i.e., Classical) required greater inhibitor quantities (Figure 6C). This result suggests that *NPC1L1* and, by extension, the cholesterol metabolism have a major role in both Basal and Classical PDAC. In addition, the combination of gemcitabine with Ezetimibe did not affect the cytotoxic effect of gemcitabine in PDAC cells (Figure S6D).

In order to study the specificity of *NPC1L1* inhibition on PDAC survival, targeted small interfering RNA (siRNA) knockdown was performed. This genetic approach revealed a dramatic effect on the survival of the siRNA-transfected cells validating the role of *NPC1L1* as an original therapeutic target (Figure 6D). The knockdown of *NPC1L1* made cells resistant to high levels of Ezetimibe, providing evidence for the specificity of the

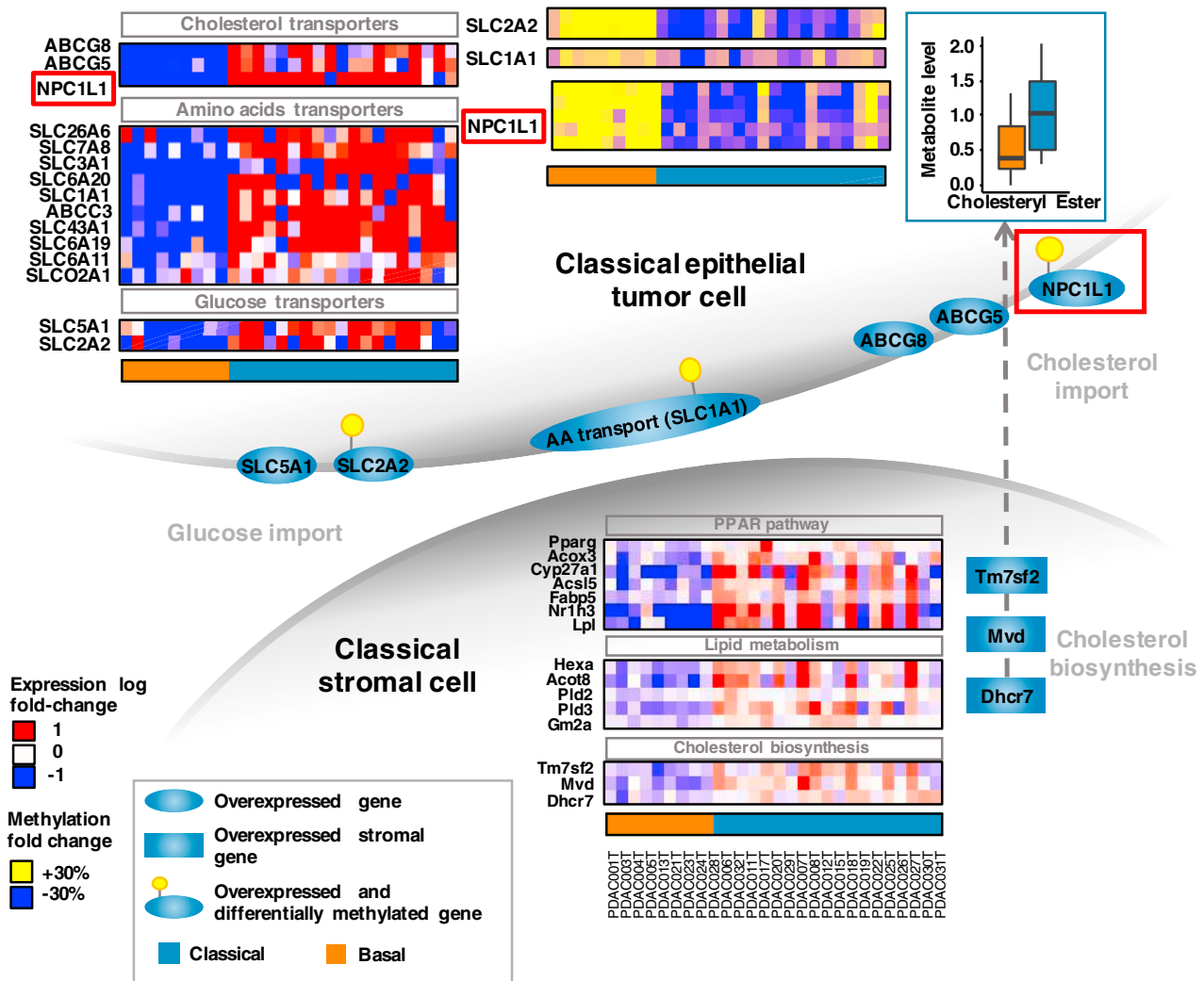


Figure 5. Classical Deregulated Tumor-Stroma Signaling Cross-Talk

Schematic illustration of the overexpressed transporters and stromal pathways in classical tumors. Ellipses represent genes overexpressed (blue) in classical PDX tumors. Yellow circles indicate methylation-associated deregulation of expression. Rectangles represent genes overexpressed in classical PDX stroma. Boxplots show the levels of cholesteryl ester in basal and classical samples. Blue-red heatmaps represent expression values of genes and miRNA. Blue-yellow heatmaps represent methylations values of CpG associated with genes. Arrows indicate the hypothetical flow of cholesterol. All heatmaps are shown with the same samples in the same order.

treatment (Figures S6E and S6F). We then derived spheroids and organoids from PDX and treated these structures with Ezetimibe for 3 and 4 days, respectively, as shown in Figures 6E and 6G. Ezetimibe treatment resulted in a significant effect on their growth, as measured by cell viability (Figures 6F and 6I) and volume change (Figure 6H). Finally, we performed a preclinical analysis by treating two PDXs with Ezetimibe (5 mg/day) in 200 μ L corn oil by a daily intraperitoneal (i.p.) injection to confirm the *in vitro* results. As shown in Figure 6J, PDAC019T (classical) and PDAC003T (basal) samples efficiently responded to the treatment by reducing their growth. Based on these *in vitro* and *in vivo* results, we conclude that *NPC1L1* is a highly effective therapeutic target for treating PDAC using Ezetimibe.

DISCUSSION

We report in this work the use of PDXs to profile both resectable as well as non-resectable PDAC. We found that PDXs obtained by biopsied PDAC are frequently associated with a more aggressive phenotype, suggesting that studies profiling PDAC still present a bias. Moreover, one advantage of a xenograft is to combine transformed tumor and stromal cells from distinct species, resulting in the ability to investigate cancer and stromal molecular profiles independently. Analysis of tumor-cell-specific molecular profiles allowed the examination of epigenetic and transcriptomic profiles, separately from non-neoplastic signals. Conversely, the absence of relationship between the consensus multiomics classification and

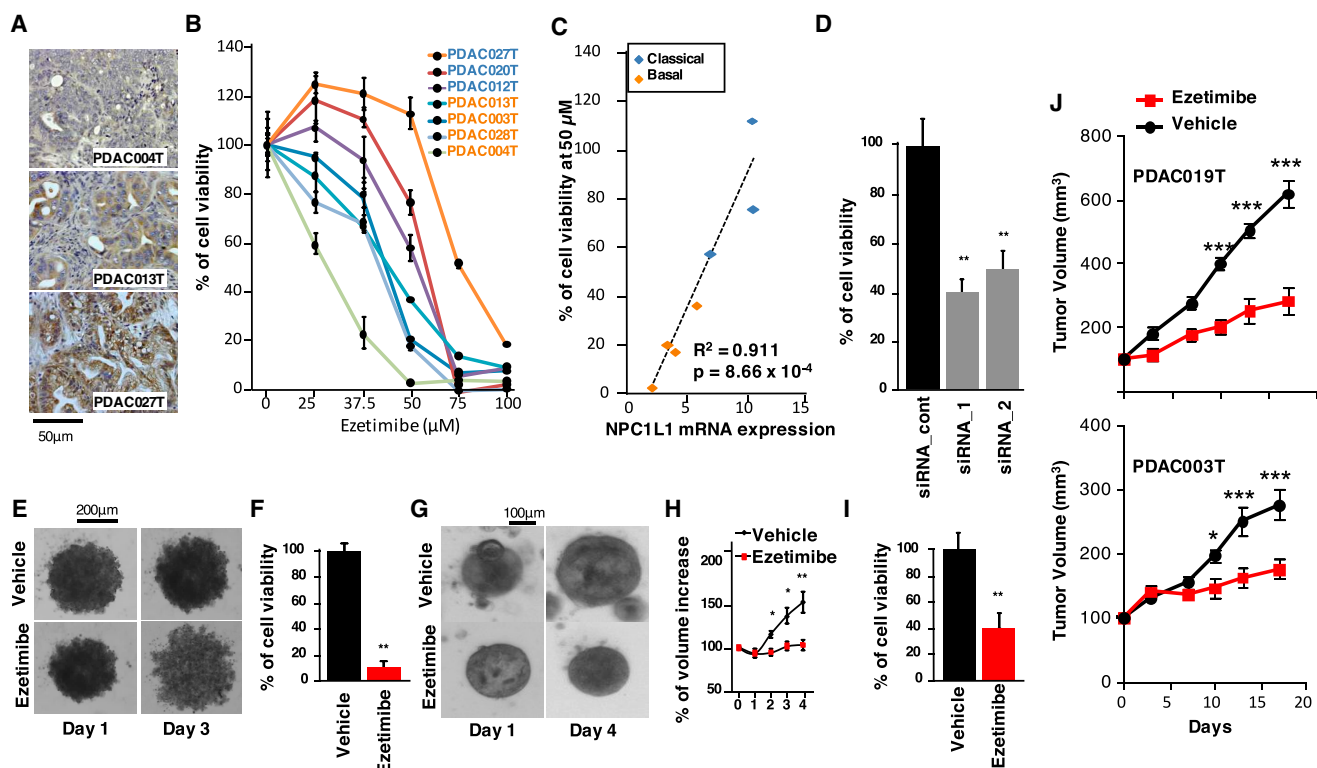


Figure 6. *NPC1L1* Is a Therapeutic Target for Treating PDAC

(A) Immunohistochemical staining of NPC1L1.
 (B) Ezetimibe sensitivity of 7 PDX-derived cell lines. Dose-response curves after 72 hr of Ezetimibe treatment. The level of cell viability is represented as a percentage to the vehicle-treated control. Error bars represent SEM; n = 6.
 (C) Correlation between survival percentage of Ezetimibe at 50 μ M and *NPC1L1* mRNA expression for the 8 PDXs with Spearman correlation.
 (D) Effect of knockdown *NPC1L1* with siRNA. siRNA_1 and siRNA_2 directed against *NPC1L1* were transfected into PDAC012T, and survival was measured 72 hr later. Data are expressed as a percentage of siRNA_control (cont)-transfected cells.
 (E and F) Effect of Ezetimibe on spheroid growth is indicated.
 (E) Representative picture of the PDAC012T-derived spheroids treated with Ezetimibe (50 μ M) or vehicle after 3 days.
 (F) Cell viability of spheroids was measured by CellTiter Glo and expressed as percentage of the vehicle-treated spheroids. **p < 0.01.
 (G–I) Effect of Ezetimibe on organoid growth is indicated.
 (G) Representative picture of the PDAC012T-derived organoids treated with Ezetimibe (50 μ M) or vehicle after 4 days.
 (H) Calculated volume of organoids treated with Ezetimibe. *p < 0.05; **p < 0.01.
 (I) Cell viability of organoids was measured by CellTiter Glo and expressed as percentage of the vehicle-treated organoids.
 (J) Two PDXs were treated with 5 mg/day of Ezetimibe or with vehicle for 17 days by i.p. injections (n = 3 per group). *p < 0.05; ***p < 0.001, in two-way ANOVA with Bonferroni post-test (mean $\pm$ SEM).

genomic alterations, which was previously proposed, is unambiguously confirmed in this work. This is a crucial confirmation, since it supports the idea of an epigenetic identity for PDAC subtypes. The most likely explanation for this observation is that genetic mutations, amplifications, and deletions are involved in the transformation process of PDAC, whereas the clinical outcome, response to treatments, and the phenotype of the tumors are controlled at the epigenetic level. This observation is of obvious clinical relevance, since emerging epigenetic drugs can be used to target these tumor characteristics and, therefore, opens a promising avenue in the treatment of PDAC patients. While preliminary genomic analysis suggested that genetic alteration could define therapeutic options (Witkiewicz et al., 2015), more recent studies found no associations between any genetic alterations and therapeutic responses

on a large number of drugs (Knudsen et al., 2017; Witkiewicz et al., 2016).

Based on the analysis of PDX, we identified two major subtypes with extensive similarities to the basal and classical human PDAC subtypes. The specific analysis of the tumor cell profiles underlined the homogeneity of basal samples while highlighting the diversity of the classical subtype. For instance, while CIMP PDACs have been described (Ueki et al., 2000), we found that this phenotype corresponds to a subdivision of classical PDAC exclusively. Overall, the two molecular subtypes describe two distinct PDAC development patterns: a dedifferentiated basal subtype, highly glycolytic and with features associated with epithelial-mesenchymal transition, and a subtype with more general digestive differentiation features reminiscent of an aberrant gastro-intestinal progenitor. In addition to having distinct and

specific signaling exchanges with their stroma, a broad homogeneity among all samples can be observed in some major tumor-stroma pathways (e.g., HGF, IGF, and Hedgehog). Despite the PDX being hosted in immunodeprived mice, we found a subgroup of samples in the classical PDX group with extensive immune infiltration that strongly correlated with the immunogenic subtype described in primary PDAC (Bailey et al., 2016). Our observations indicate that this subgroup is broadly driven by its stromal content and is, in fact, a “classical inflammatory infiltrated” subtype instead of a specific subtype by itself. Although these models are not suitable to study immune therapy, these results show the extent to which xenografts can reproduce primary PDAC phenotypes, including interaction with the immune microenvironment. Importantly, athymic NMRI nude mice (*Foxn1nu*) lack conventional T cell development, but this does not preclude extrathymic maturation such as intestinal T cell differentiation, which likely explains the extensive infiltration of the immunogenic-like PDX. Although nude mice lack thymus-dependent lymphocytes, other immune cell populations such as macrophages, natural killers and B cells are present and functional. Given the potential of PDX to reproduce an immunogenic PDAC subtype in a model with an only partial immune landscape, new developments in animal models, in particular humanized mice, can be expected to greatly improve the present model and potentially be used to investigate immune therapies.

In addition to greatly improving the multiomics description of tumor-specific altered pathways, the precise distinction of tumor and stromal cells in the same samples enabled analysis of cross-talk at the genome-wide level. The simple description of the most expressed genes in each compartment highlighted known molecular dialogs between tumor cells and their stroma. The role of PDAC stroma on tumor survival and growth is not settled. It was previously suggested that the stroma plays a major role in the resistance to the PDAC treatments, because it acts as a mechanical barrier impeding the access of the drugs and also inhibits the formation of vessels. Therefore, it seemed important to combine agents against cancer cells with compounds targeting the stroma to improve efficiency. Accordingly, recent results encourage going forward in this way. In fact, initial testing of the Hedgehog signaling pathway inhibitor IPI-926 demonstrated a promising transient effect on the tumor microenvironment, leading to improved drug delivery and an association with improved survival in mice treated with both IPI-926 and gemcitabine (Olive et al., 2009). Mouse models of PDAC deficient in Sonic Hedgehog (Rhim et al., 2014) demonstrated that Hedgehog inhibition leads to an absence of tumor stroma and an abundance of intratumoral blood vessels yet increases tumor aggressiveness and metastasis. Another potential stroma-targeting therapy relates to glycosaminoglycan hyaluronan, which is overrepresented in PDAC stroma and which can be degraded by hyaluronidase. Notably, treatment with PEGPH20, a PEGylated human recombinant hyaluronidase, induces a rapid perfusion increase, leading to the inhibition of growth when combined with chemotherapy in mouse PDAC models (Walker et al., 2015). While the outcome of stroma-targeting therapies remains unknown, major signaling pathways driving PDAC have been shown to be stimulated by the non-transformed cells composing its microenvironment. Our

work revealed the potential sustaining role of the stroma as subtype specific. Indeed, the analysis of the recruited stroma diversity revealed the unidentified subtype specificity of the stroma through the discovery of convergent tumor/stromal classifications, which was verified in four different datasets of primary tumors. This is, to some extent, dissimilar to the activated stroma previously described (Moffitt et al., 2015) as subtype independent. Overall, our results show that the stroma, originating from surrounding host cells, is closely associated with the phenotype of the tumor indicating that the composition and function of the tumor microenvironment may be trained by tumor cells.

A major advantage of the PDX model is that it is a perpetual source of living material conserving original biological characteristics. Indeed, each PDX can be molecularly analyzed and concomitantly used for testing biological hypotheses or putative therapeutic targets derived from these analyses. In this work, we describe several subtype-specific pathways, thereby highlighting potential targeted therapies. We used *NPC1L1*, a transporter of cholesterol, as a proof of concept of an original candidate to be targeted for treating PDAC. The function of this gene can be inhibited by a specific inhibitor, Ezetimibe, which has been routinely used for the treatment of hypercholesterolemia. While no studies describe the association of *NPC1L1* or its inhibitor with pancreatic cancer, statins (used for the treatment of hypercholesterolemia) were shown in epidemiological studies to reduce the risks of pancreatic cancer (Walker et al., 2015) and to improve survival (Huang et al., 2016; Wu et al., 2015). Preclinical tests demonstrated that using the specific inhibitor Ezetimibe or genetic knock-down approaches results in a dramatic effect on the survival capacity of PDAC cells and on the growth of spheroids and organoids *in vitro* and of PDX *in vivo*. Finally, we also demonstrated that Ezetimibe treatment does not affect the cytotoxicity of gemcitabine, suggesting that patients could be treated with an inhibitor of the *NPC1L1* transporter in combination with a conventional anticancer drug without influencing its effect. Altogether, our data demonstrate that *NPC1L1* is an interesting and promising therapeutic target.

In conclusion, the data presented in this work reveal that PDX is a suitable model for preclinical studies, representing the diversity of the primary cancers in which the stroma is reconstituted. Its multiomics analysis is a rich source of novel and reliable therapeutic targets for treating patients with PDAC.

EXPERIMENTAL PROCEDURES

PDXs

Three expert clinical centers collaborated on this project after receiving ethics review board approval. Patients were included in this project under the Paoli-Calmettes Institute clinical trial number 2011-A01439-32. Consent forms of informed patients were collected and registered in a central database. The tumor tissues used for xenograft generation were deemed excess to that required for the patient's diagnosis. PDAC tissue from surgical samples was fragmented, mixed with 100 μ L Matrigel, and implanted with a trocar (10G; Innovative Research of America, Sarasota, FL) in the subcutaneous right upper flank of an anesthetized and disinfected male NMRI-nude mouse. Samples obtained from endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) were mixed with 100 μ L of Matrigel (BD Biosciences, Franklin Lakes, NJ) and injected in the upper right flank of a male nude mouse (Swiss Nude Mouse Crl:NU(lco)-Foxn1nu; Charles River Laboratories, Wilmington, MA) for the first implantation. When xenografts reached 1 cm³, these were removed

and passed to NMRI-nude mice in the same manner as surgical samples. In total, 30 xenografts from 29 different patients were generated, and early passages were used for large-scale molecular profiling.

Genome-wide Profiles and Data Availability

Genotype data from whole-exome sequencing have been deposited at the European Genome-phenome Archive (EGA; <http://www.ebi.ac.uk/ega/>), which is hosted by the EBI, under accession number EMBL-EBI: EGAS00001001928. Other datasets are available on ArrayExpress (<http://www.ebi.ac.uk/arrayexpress/>): SNP data are available under accession number EMBL-EBI: E-MTAB-5006, Methylation data are available under accession number EMBL-EBI: E-MTAB-5008, miRNA sequencing data are available under accession number EMBL-EBI: E-MTAB-5018, and mRNA sequencing data are available under accession number EMBL-EBI: E-MTAB-5039. Sequencing datasets were processed using SMAP (see [Supplemental Experimental Procedures](#)) to separate mouse and human reads. In addition to raw data, all processed data produced by and used in this study are available through our institutional web portal (<http://cit.ligue-cancer.net/pacaomicsdata-web-page>), and simple gene-level queries for expression and methylation are available using a custom application (<http://cit-apps.ligue-cancer.net/pdac.pacaomics>).

Public Dataset Comparison

ICGC methylation chips, RNA-seq, and microarray gene expression datasets were downloaded from the ICGC data portal (dcc.icgc.org, release 20). TCGA data were downloaded through the Broad Institute TCGA Genome Data Analysis Center (GDAC) firehose tool (gdac.broadinstitute.org, 20160411 data snapshot). Other datasets were downloaded from the provided GEO entries GSE171729 (Barr et al., 2012, 2010; Moffitt et al., 2015) and GSE17891 (Collisson et al., 2011; Martínez-Arranz et al., 2015; Nones et al., 2014). All non-cancer samples were removed from each dataset. Expression datasets were then centered gene-wise. Centroid classifiers were built for each dataset defining a classification using a Pearson-based approach described in previous work (Bailey et al., 2016; Guinney et al., 2015; Marisa et al., 2013).

Primary Cell Cultures and Sensitivity

Primary cell cultures were obtained from PDX. Ezetimibe was obtained from Selleckchem (ref. S1655). Tissues were split into several small pieces and processed in a biosafety chamber. Five thousand cells per well were plated in 96-well plates in serum-free defined media (SFDM) medium. The RNA profile of each cell culture showed a contamination in murine cells of less than 3%. Twenty-four hours later, the media were supplemented with increasing concentrations of Ezetimibe and incubated for an additional 72-hr period. Cell viability was estimated with the PrestoBlue (Invitrogen, Grand Island, NY) reagent. Each experiment was done in triplicate and repeated at least three times. Six increasing concentrations of Ezetimibe were used ranging from 0 μ M to 100 μ M.

Spheroid Sensitivity to Ezetimibe

Fifteen thousand cells per well were seeded in 96-well round-bottom plates with medium containing 20% methylcellulose (Sigma-Aldrich, St. Louis, MO, USA). After 48-hr incubation, cells with spheroids of uniform size and shape were incubated with 50 μ M Ezetimibe during 72 hr. Spheroid growth was monitored for 96 hr by taking microphotographs every day or assessed with the CellTiter-Glo 3D kit at the end of the treatment according to the manufacturer's instructions (Promega, Madison, WI). Values were normalized and expressed as the percentage of the control treatment (DMSO, 0.05%).

Pancreatic Organoid Generation and Treatment with Ezetimibe

Organoid suspensions obtained from fresh tissue samples were placed into a 12-well plate coated with 100 μ L GFR Matrigel. Media were replaced every 2–3 days. Twenty-four hours after seeding, organoids were incubated with 100 μ L pancreatic organoid feeding media (POFM) supplemented with 50 μ M Ezetimibe or 100 μ L POFM supplemented with vehicle (0.5% DMSO) into 96-well plates coated with 15 μ L Matrigel GFR. Organoid growth was monitored for 96 hr by taking microphotographs every day or assessed with the CellTiter-Glo 3D kit at the end of the treatment. Values were normalized and expressed as the percentage of the control.

Treatment with Ezetimibe of PDX *In Vivo*

We transplanted two PDX samples (PDAC019T and PDAC003T) subcutaneously into 6-week-old male Swiss nude mice (CrI: Nu(lco)-Foxn1nu; Charles River Laboratories, Wilmington, MA). Each PDX sample was inoculated into six nude NMRI mice that were randomized for treatment ($n = 3$) and vehicle ($n = 3$). When the PDX reached 100 mm³, we started the treatment with Ezetimibe or vehicle and followed their growth for 17 days. Ezetimibe was prepared as a solution of 250 mg/mL in ethanol and then resuspended in corn oil at 25 mg/mL, and 200 μ L was administered in i.p. injections every day. Tumor size was measured with a Vernier caliper twice weekly, and the tumor volume was calculated with the equation $v = (\text{length}/\text{width})^2/2$.

DATA AND SOFTWARE AVAILABILITY

The accession number for exome sequencing reported in this paper is EMBL-EBI: EGAS00001001928. The accession number for SNP arrays reported in this paper is EMBL-EBI: E-MTAB-5006. The accession number for the ethylation array reported in this paper is EMBL-EBI: E-MTAB-5008. The accession numbers for miRNA-seq and RNA-seq reported in this paper are EMBL-EBI: E-MTAB-5018 and E-MTAB-5039, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and four tables and can be found with this article online at <https://doi.org/10.1016/j.celrep.2017.11.003>.

AUTHOR CONTRIBUTIONS

C.L., O.G., B.B., M.B., L.A., M.A., P.D., A.M., and J.G. performed the biological experiments and sample preparation. M.A. and N.E. conducted data management. M.R., V.S., S.G., J.R., and F.P. performed the histochemical stainings and immunohistochemical scoring. V.M., O.T., M. Giovannini, M. Gasmi, M.O., J.E., E.B., A.G., J-L.R., P.G., M.B.-B., E.N., M. Gilabert, F.G., S.V., and J.-R.D. provided material. R.N., Y.B., and L.M. performed bioinformatics analysis. R.N., Y.B., L.M., G.L., J.-M.B., E.C., R.U., A.d.R., N.D., and J.I. contributed to the experimental design, data analysis and discussion. A.d.R., N.D., and J.I. directed the project. R.N., Y.B., L.M., N.D., and J.I. wrote the manuscript.

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A subgroup of pancreatic adenocarcinoma is sensitive to the 5-aza-dC DNA methyltransferase inhibitor

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ABSTRACT

Pancreatic Ductal Adenocarcinoma (PDAC) is a disease with a great heterogeneity in the response to treatments. To improve the responsiveness to treatments there are two different approaches, the first one consist to develop new and more efficient drugs that intent to cure all patients and the second one is to use already-approved drugs, alone or in combination, but selecting beforehand the most sensitive patients. In this work we explored the efficiency of the second possibility. We developed a collection of 17 PDAC samples collected by Endoscopic Ultrasound-Guided Fine-Needle Aspiration (EUS-FNA) or surgery and preserved as xenografts and as primary cultures. This collection was characterized at molecular level by a transcriptomic analysis using an Affymetrix approach. In this paper we present data demonstrating that a subgroup of PDAC responds to low doses of 5-aza-dC. These tumors show a specific RNA expression profile that could serve as a marker, but there is no correlation with *Dnmt1*, *Dnmt3A* or *Dnmt3B* expression. Responder tumors corresponded to well-differentiated samples and longer survival patients. In conclusion, we present data obtained with the well-known drug 5-aza-dC as a proof of concept that a drug that seems to be inefficient in solid tumors in general could be applicable to a particular subgroup of patients with PDAC.

INTRODUCTION

Pancreatic adenocarcinoma (PDAC) is the fourth leading cause of cancer death, with a median survival of 6 months and a dismal 5-year survival rate of less than 5%. Importantly, PDAC could move up to the second place as cause of cancer death as early as 2020 according to a new report from the Pancreatic Cancer Action Network. Moreover, using information from the Surveillance Epidemiology and End Results database, in 2030 the

number of patients with a PDAC will represent more than a two-fold increase over the current rate in the occidental world [1]. Based on these data the number of deaths from PDAC will exceed those from breast and colorectal cancer, and will be surpassed only by the loss of life from lung cancer.

PDAC is one of the most intrinsically drug-resistant tumors and resistance to chemotherapeutic agents is a major cause of treatment failure. Gemcitabine is the standard chemotherapeutic drug for patients

with advanced pancreatic cancer after a phase III trial performed in 1997 that demonstrated a modest survival advantage of this agent over 5-FU. Surprisingly, the most important improvement associated to this treatment was alleviation of disease-related symptoms [2]. More recently, a polychemotherapy regimen combining 5-FU, irinotecan, and oxaliplatin (FOLFIRINOX) was shown to nearly double overall survival compared to gemcitabine, at the expense of a manageable but increased toxicity, limiting its use to good performance status patients. Nevertheless, overall survival was less than 12 months [3]. Therefore, there is a dire need for designing new and targeted therapeutic strategies that can overcome the drug-resistance and improve the clinical outcome for patients diagnosed with pancreatic cancer.

Notably, the great heterogeneity in the response to treatments between patients could be based not only on the differences between hosts but also between PDAC intrinsic characteristics, indicating the existence of many PDAC sub-types. For example, the objective response rate was 31.6% in the multidrug FOLFIRINOX protocol and only 9.4% for patients treated with gemcitabine showing that near to 70% and 90% of patients are not responders respectively [3]. This responsiveness to the treatment is not predictable except for the unresponsiveness to the Gemcitabine treatment in the absence of the specific transporter hENT1 expression [4, 5]. Two different and opposite approaches could be developed to improve the responsiveness to the treatments, the first one consists in developing new and more efficient drugs intending to cure all patients. This approach is extremely expensive and the result uncertain. The second one is based on already-approved drugs, used alone or in combination, selecting beforehand the most sensitive patients. This option presents many advantages including the fact that drugs are easily available, low costs of treatment, toxicity is well known, and the preclinical trials almost unnecessary. In agreement with this second approach, we developed a collection of 17 PDAC samples from 17 consecutive patients of whom we have the complete clinical outcome. The tumors were preserved as xenografts and as primary cultures allowing the growth of cancerous epithelial cells only. This collection, which was well characterized at the molecular level, serves us to test the tumor sensitivity to different known drugs and as an important source of molecular markers.

DNA methylation is an epigenetic modification that maintains DNA transcriptionally quiescent causing gene silencing. DNA methyltransferases (DNMTs) are the enzymes that catalyze the addition of methyl groups to the 5' carbon of the cytosine residues [6]. Several isoforms of DNMTs are present in cells. DNMT1 is associated to the maintenance of established patterns of methylated DNA, while DNMT3A and DNMT3B seem to mediate de novo DNA methylation patterns [7, 8]. Many different DNMT inhibitors have been developed (nucleosides

analogues such as Azacitidine, Decitabine and Zebularine or non-nucleosides analogues such as MG98, RG108 and Procainamide) and multiple molecular mechanisms by which DNMT inhibitors induce anti-cancer effects have been identified, in most cases by the modulation of specific genes involved in cellular processes such as apoptosis, cytostasis, differentiation and tumor angiogenesis. Therefore, it is not surprising that DNMT inhibitors are emerging as promising class of drugs in cancer treatment, especially in combination with other agents or with other treatments.

5-aza-dC is a DNMT inhibitor incorporated into DNA as a deoxycytidine analog that forms irreversible covalent bonds with DNMT at cytosine sites targeted for methylation [9]. 5-aza-dC demonstrates activity against hematologic malignancy [10] and is used as first-line of treatment in AML patients > 65 years who are not candidates for intensive chemotherapy [11], whereas its efficacy in solid tumors is very limited [12]. Clinical responses appear to be exerted both by epigenetic alterations and by induction of cell-cycle arrest and/or apoptosis [13, 14]. In this paper we present data demonstrating that a subgroup of PDAC responds to low doses of 5-aza-dC, these tumors show a specific RNA expression profile that could serve as a marker, but there is no correlation with *Dnmt1*, *Dnmt3A* or *Dnmt3B* expression. Responder tumors corresponded to well-differentiated samples and longer survival patients. In conclusion, we present the data obtained with the well-known drug 5-aza-dC as a proof of concept that a drug that seems to be inefficient in solid tumors in general could be applicable to a particular subgroup of patients.

RESULTS

Chemogram to the 5-aza-dC compound

Primary cell cultures from patient's pancreatic tumors were submitted to increasing concentrations (from 0 to 80 μ M) of 5-aza-dC in order to study their sensitivity and to obtain a dose-response curve. Using this approach we were able to compare 17 different PDAC-derived primary cultures estimating their relative chemosensitivity. As shown in Figure 1A and Table 1, each patient-derived primary culture shows a different pattern of chemosensitivity. Whereas A-NOR (DL50 = 0.29 μ M), D-IPC (DL50 = 0.29 μ M) and 01.030 (DL50 = 0.50 μ M) are the 3 most sensitive patient derived primary cultured cells to 5-aza-dC; the Foie_8b (DL50 >80 μ M), L-IPC (DL50 = 41.0 μ M) and H-NOR (DL50 = 33.0 μ M) are the most resistant to the 5-aza-dC compound. The sensitivity of two resistant primary cultures (L-IPC and Foie-8b) and two sensitive (HN-01 and B-TIM) were tested *in vivo* and results showed in Figure 1B. To this end, four tumors (100 mm³) corresponding to two sensitive (HN-01 and B-TIM) and two resistant (L-IPC and Foie_8b) primary

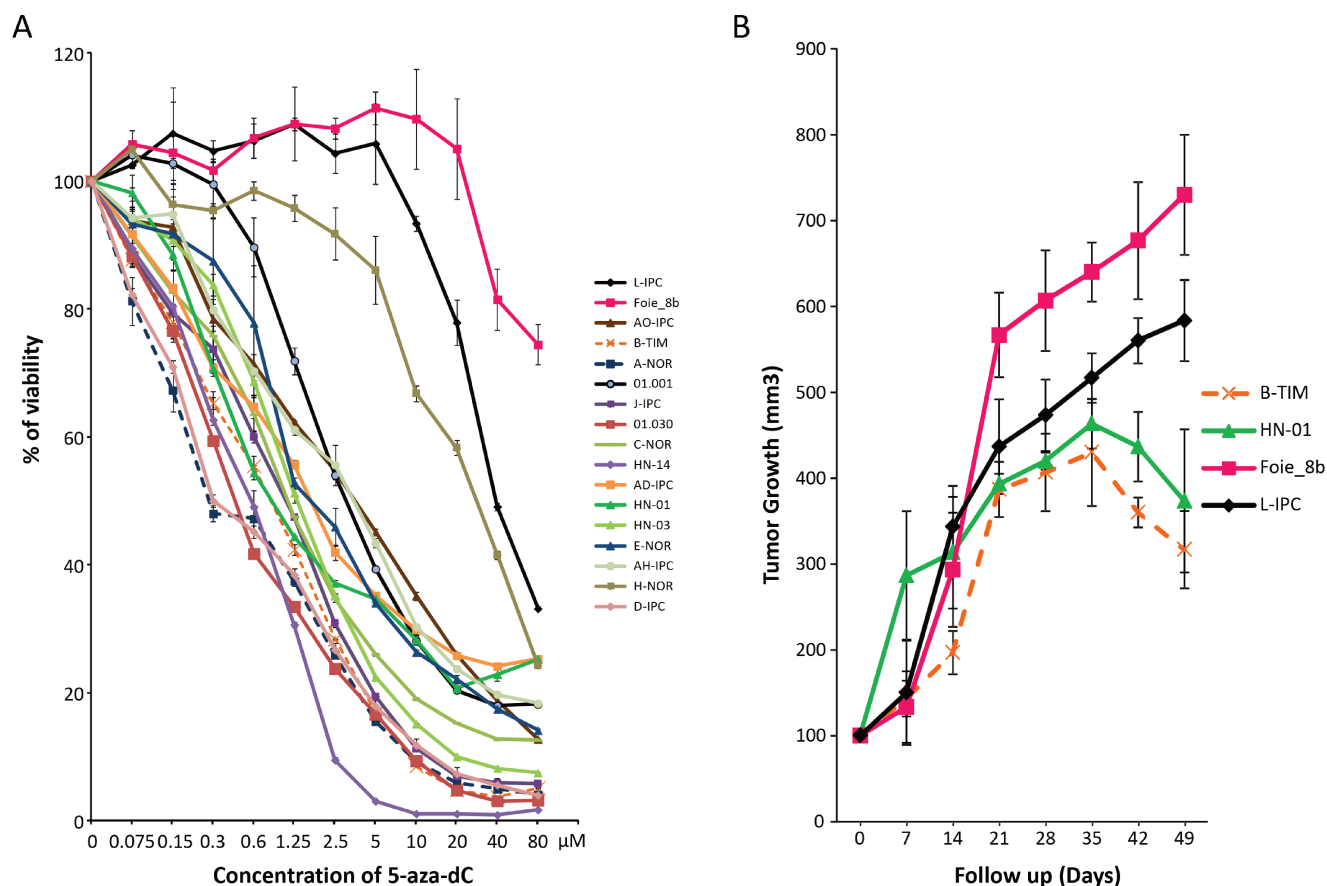


Figure 1: Sensitivity to the 5-aza-dC *in vitro* and *in vivo*. (A) Chemogram of 5-aza-dC. PDAC-derived cells were treated with increasing concentrations of 5-aza-dC and the survival cells were measured after 72 h of treatment. (B) *in vivo* 5-aza-dC sensitivity. PDAC xenografted mice were treated with 5-aza-dC and tumor measured weekly. Treatment starts at day 21 after implantation. In grey is marked the length of the treatment. Error bars $\pm$ SEM; $n = 3$ per group.

cultures were xenografted in nude mice. Treatment with 5-aza-dC was started at day 21 after implantation and for a period of 4 weeks. Tumor growth curves confirm that L-IPC and Foie-8b xenografts are 5-aza-dC resistant tumors whereas HN-01 and B-TIM xenografts are more sensitive in agreement with data obtained *in vitro*.

Altogether, these results show that PDAC-derived primary cultures present variable sensitivities to 5-aza-dC with DL50s ranging from 0.29 to $> 80 \mu\text{M}$ which is a range of more than 320 folds and that *in vitro* experiments correlates with the *in vivo* treatments. This strong variability encourages us to go forward with this study trying to find molecular markers that may identify sensitive patients.

Correlation between transcriptome and 5-aza-dC sensitivity in PDAC

To study whether or not there is a correlation between 5-aza-dC response and transcriptome, we performed a heatmap analysis on the transcriptome

of xenografted human PDAC by clustering tumors according to their expression profiles. To our surprise, the 5 more resistant PDAC (Foie_8b, L-IPC, H-NOR, AO-IPC and 01.001) and the moderately resistant HN-03 (DL = $1.30 \mu\text{M}$) appear in a relatively homogeneous group of tumors whereas the other 12 more sensitive PDAC form a relatively distant group of tumors as shown in Figure 2 and Supplementary Tables 1 and 2.

Among the transcripts identified in this phenotype, 326 corresponded to upregulated whereas 381 to downregulated genes involved in several pathways. Among these data, we observed that resistant tumors are undifferentiated or poorly differentiated contrary to the sensitive tumors that are well or middle differentiated. We observed that TGFB1 and its target gene TGFBI are overexpressed in resistant tumors by 3.24 and 3.08 fold. Their strong involvement in epithelia to mesenchymal transition process may explain the accrued resistance to the treatment.

Altogether, these results suggest that a specific tumor phenotype could be associated with resistance or sensitivity to an anticancer drug which could be of clinical interest.

Table 1: DL50 values corresponding to 17 pancreatic cancer-derived cells

Cell	DL50
01.001	3.10
01.030	0.50
AD-IPC	1.80
AH-IPC	3.80
A-NOR	0.29
AO-IPC	3.90
B-Tim	0.90
C-NOR	1.26
D-IPC	0.29
E-NOR	1.83
Foie_8b	>80
H-N01	0.91
H-N03	1.30
H-N14	0.63
H-NOR	33.0
J-IPC	0.58
L-IPC	41.0

Sensitivity to 5-aza-dC in PDAC does not correlate with expression of *Dnmt1*, *Dnmt3A* or *Dnmt3B*

Because, on one hand, the 5-aza-dC is an inhibitor of the DNMT1 activity and, on the other hand, it was proposed a relationship between sensitivity to the drug

and DNMT1 expression in PDAC cells, we analyzed the correlation between DNMT1 as well as DNMT3A- and DNMT3B-related enzymes with the sensitivity of the cells to the treatment with 5-aza-dC. To this end we measured the expression of all this three transcripts in the RNA purified from the xenografts and we correlated these data with the DL50 obtained on the xenograft derived primary cultures. As shown in Figure 3, the R^2 for DNMT1 is 0.0555, for DNMT3A is 0.0021 and for DNMT3B is 0.0028 which are extremely low indicating no correlation. These results strongly suggest that the sensitivity to 5-aza-dC is independent of the DNMTs expression levels and therefore measuring their levels is clinically irrelevant.

Correlation between sensitivity to the 5-aza-dC and the clinical outcome

From a histopathological point of view, we observed that resistant tumors are undifferentiated or poorly differentiated and that sensitive tumors are well or moderately differentiated (Figure 4A). The sensitivity curve to the 5-aza-dC treatment was obtained from 17 primary cultured cells derived from the xenografts and a putative correlation with the clinical outcome of patients was analyzed. Data presented in Figure 4B indicate a modest correlation with the survival time of patients. These results are in agreement with the fact that patients with undifferentiated and poorly differentiated tumors have a shorter survival time as presented in Figure 4C. These observations suggest that patients with a well differentiated PDAC should be more sensitive to the treatment with 5-aza-dC and probably with other DNMTs inhibitors. Altogether, our results suggest that because of

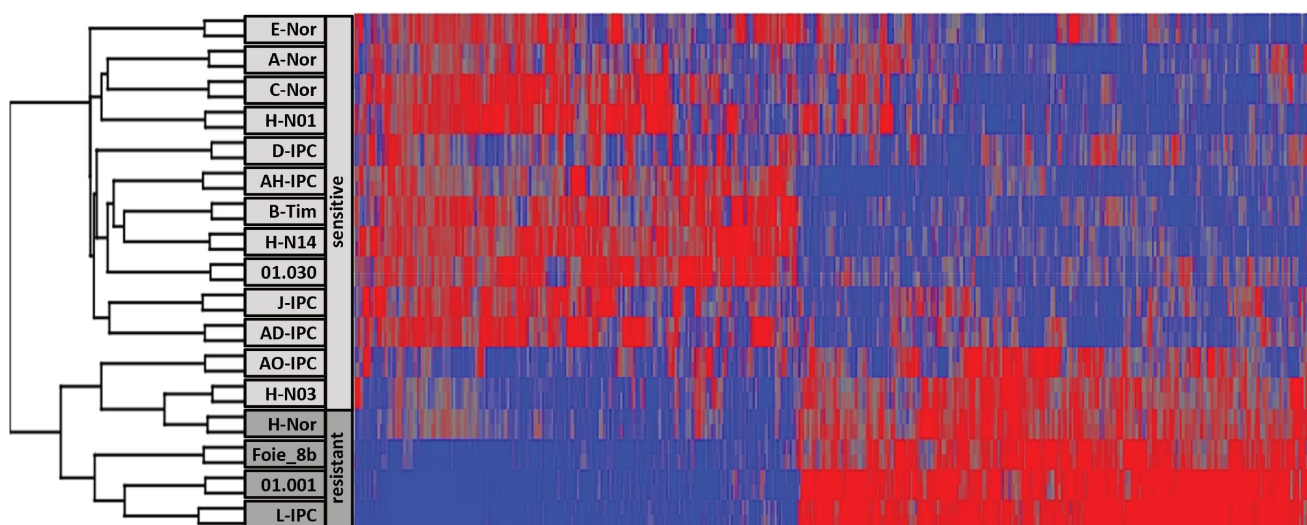


Figure 2: RNA expression analysis of PDAC. The Heatmap showing the RNA expression profile of PDAC sensitive and resistant to the 5-aza-dC treatment.

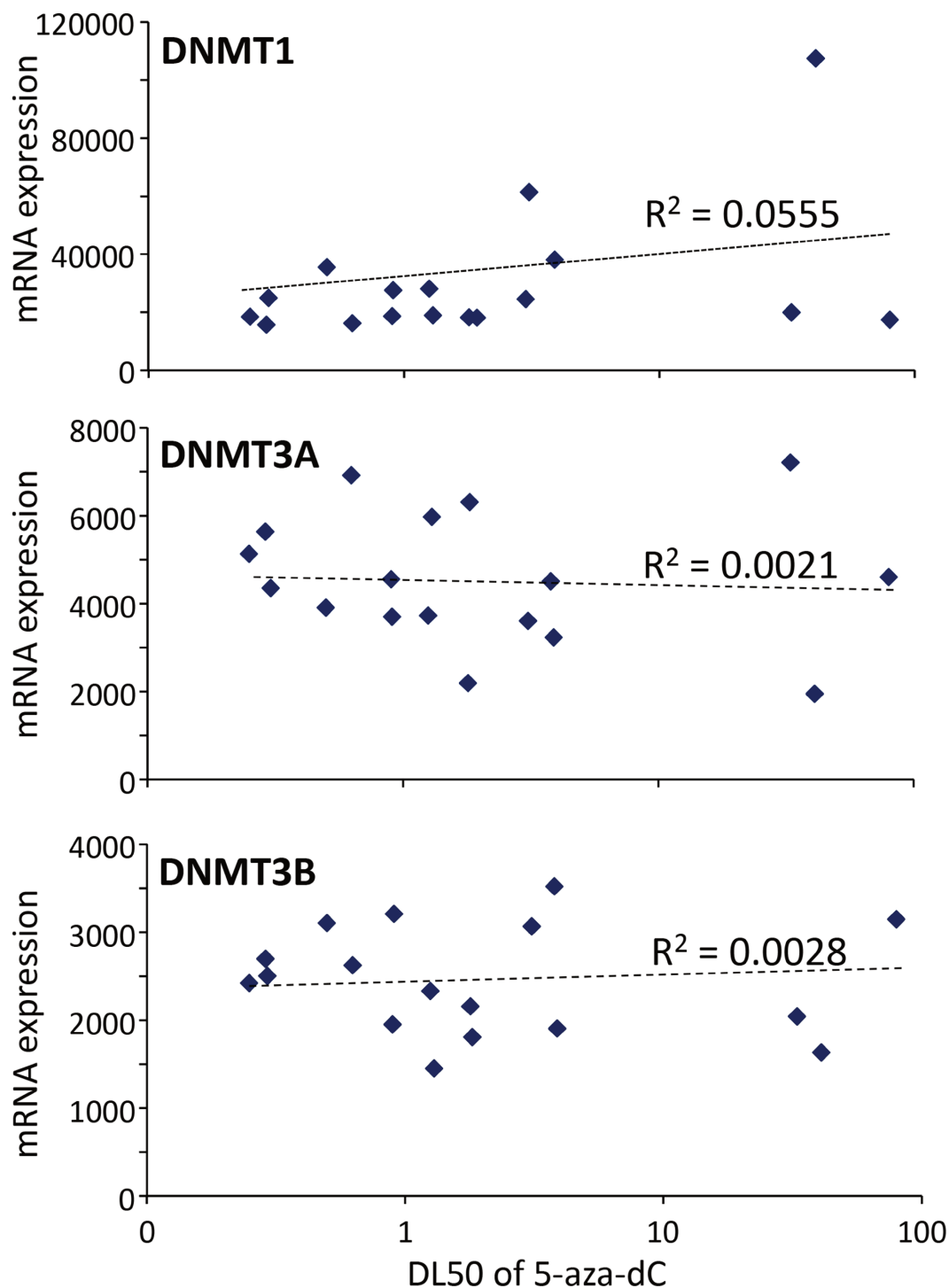


Figure 3: Correlation between sensitivity of the 5-aza-dC and expression of DNMT1, DNMT3A and DNMT3B transcripts. DL50 of 5-aza-dC was calculated for each xenograft-derived cell and correlated with expression of DNMT1, DNMT3A and DNMT3B mRNAs. r^2 is the correlation coefficient.

the heterogeneity of PDAC, a subgroup of patients with well differentiated tumors, are more sensitive to 5-aza-dC than undifferentiated tumors. An additional work including a larger number of patients will be necessary to

confirm this observation, however, these results validate the concept according to which studying the sensitivity of PDAC derived cells to a set of drugs could allow to define a line of treatment more adapted to the patient.

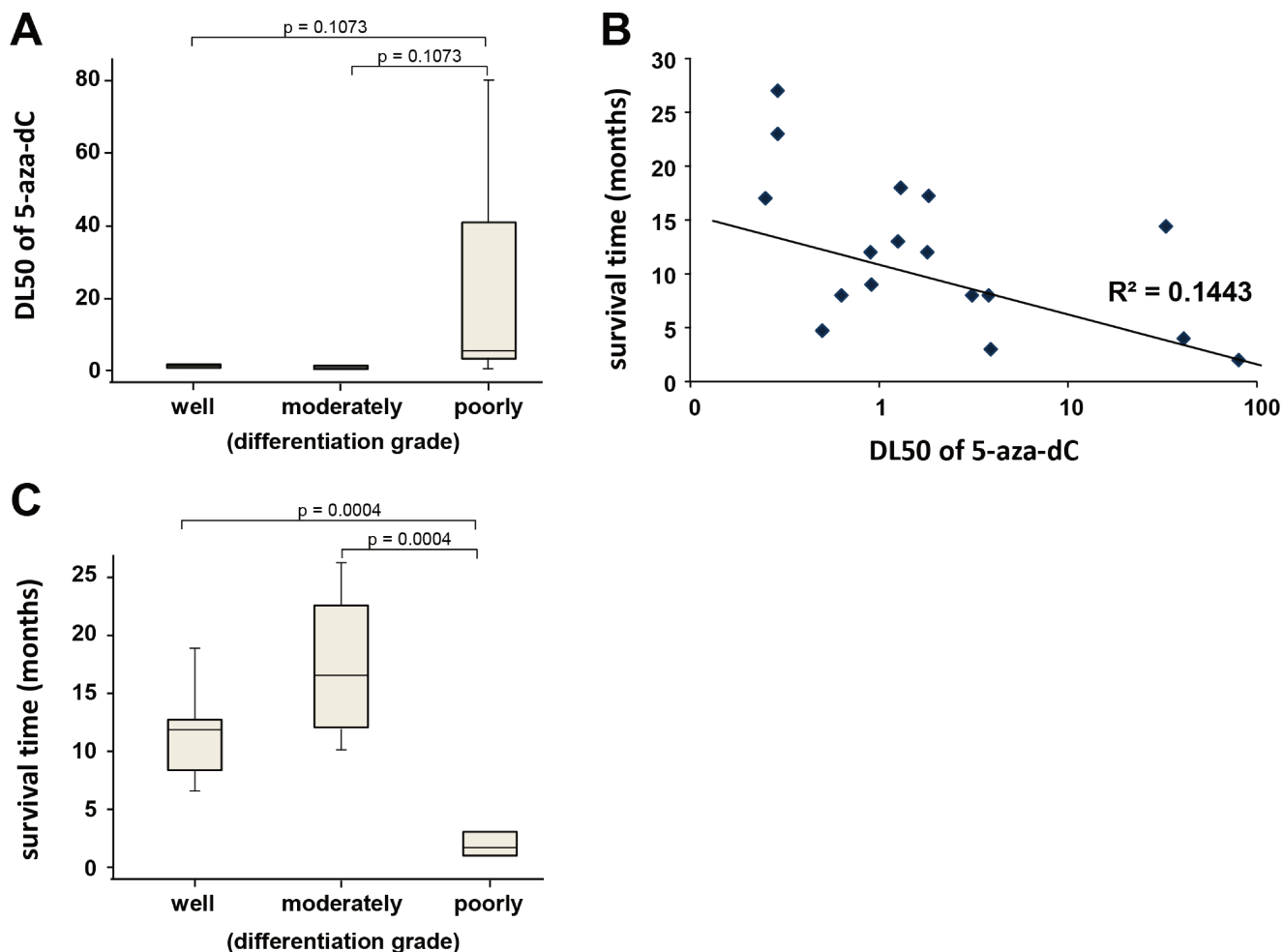


Figure 4: (A) Box and whiskers plot showing the distribution of DL50 for 5-aza-dC in a three point score of tumor differentiation (well, moderately and poorly differentiated or undifferentiated). (B) Correlation between sensitivity of the 5-aza-dC and survival time of patients. DL50 of 5-aza-dC was calculated for each xenograft-derived cell and correlated with the time of survival for each patient in months. r^2 is the coefficient of correlation. (C) Box and whiskers plot showing the distribution of patient's survival time in a three point score of tumor differentiation.

DISCUSSION

The main goal of this study was to define whether there are differences in PDAC sensitivity to drugs and in such case, if there is a possibility to identify the most sensitive patients by using specific markers that arise from the molecular characterization of their tumors. The results presented in this paper suggest that there is a group of tumors sensitive to the well-known drug 5-aza-dC. 5-aza-dC is an interesting compound since its mechanism of action is well known and several clinical trials have been performed in patients with different tumors, including several solid tumors.

The rationale to use methyltransferases inhibitors to treat tumors is that neoplastic cells exhibit global hypomethylation with localized hypermethylation of CpG

islands and increased levels of methyltransferases activity [15]. Moreover, aberrant hypermethylation of CpG islands is associated with transcriptional silencing of genes, which not only plays a role in tumorigenesis, but may also influence response to anticancer agents [16, 17]. Therefore, reversing gene methylation and epigenetic silencing has the potential to influence tumor growth, sensitivity to anticancer agents, and ultimately clinical outcome [18]. Several studies have documented the relevance of epigenetic alterations in pancreatic cancer and the effect of 5-aza-dC on cell coming from this tumor [19–21]. In clinical trials, although the 5-aza-dC has shown an objective response in some patients, its overall efficiency remains relatively low. For these reason 5-aza-dC is not used in the treatment of pancreatic cancers. This is a proof of concept study that intent to demonstrate that drugs that

present an interest in a particular sub-group of patients should be studied by identifying the sensitive tumors with specific markers.

Surprisingly, there is no correlation between sensitivity to 5-aza-dC and DNMT1, neither with DNMT3A or DNMT3B, expression level but a significant correlation was observed with an expression profile specifically associated to sensitivity. In addition, expression of other DNA methylation associated molecules such as Mecp2 (methyl CpG binding protein 2) or Polycomb-group proteins including SUZ12, Eed, Ezh1 and Ezh2 do not correlate with the 5-aza-dC sensitivity (data not shown). These results are interesting and original because they show that effect of the drug is not dependent of its target level and they indicate that sensitivity is dependent on other cellular mechanisms. This is not surprising since there are no reports in the literature associating efficiency of 5-aza-dC and levels of DNMT1 expression in tumors except data obtained by Li and colleagues which conclude that PDAC-derived cells with low DNMT1 expression tend to be more sensitive to low dose of 5-aza-dC [22]. Altogether, these results strongly suggest that there is no correlation or if any it is only small.

One of the most interesting points from this work is the fact that we used an efficient strategy in which PDAC tumors from 17 consecutive patients were collected from surgical specimens and EUS-FNA biopsies by xenografting in immunosuppressed mice. We studied the RNA expression profile by a microarray approach on these xenografts, rather than in the cultured cells, since morphology and microenvironment, both playing determinant effect on RNA expression, were closer to the original human PDAC as recently confirmed [23]. Then, primary culture of cells obtained from xenografts allowed us to analyze their relative sensitivity to 5-aza-dC *in vitro*. All these data were used to detect a possible correlation between drug responsiveness and RNA expression profile. Surprisingly, we identified a profile of genes which significantly correlates with the sensitivity to the treatment suggesting that the profile of expression can be used to identify tumors types and thereby establish groups of tumors with more or less sensitivity to the drug. We are aware that the number of genes we found defining sensitivity to 5-aza-dC treatment is quite high but after increasing the number of PDAC samples will probably allow us to detect the most efficient and robust genes. Therefore, we can expect that increasing the number of patients will increase the sensitivity and specificity with a more limited number of genes to constitute the signature determining drug sensitivity and consequently patient outcome.

Another interesting point to be noted is that sensitivity to 5-aza-dC treatment correlates with the long-term survival patients carrying well- and moderately-differentiated tumors. This is in agreement with the fact that

some genes typically expressed in poorly-differentiated PDAC such as MUC3A (13.3 fold increase), MUC5AC (9.76 fold increase), GATA6 (5.57 fold increase), or HNF4A (4.43 fold increase) are differentially overexpressed in sensitive compared to resistant PDAC-derived cells (Supplementary Table 2). This data strongly suggest that 5-aza-dC treatment is more efficient against well- and moderately-differentiated tumors than against the poorly-differentiated ones. Interestingly several studies have shown that after resection of pancreatic cancer, a measure of the tumor differentiation degree is an important prognostic indicator, in general, the more undifferentiated the tumor, the more aggressive the malignant biology [24].

In conclusion, we showed in this work that expression profiling of xenografts from PDAC patients is able to discriminate sensitive from resistant tumors. This fact should be used to aid to take decision to treat patients with a PDAC.

METHODS

PDAC samples and cell culture

Consent's forms of informed patients were collected and registered in a central database. The tumor tissues used for xenograft development was deemed excess to that required for the patient's diagnosis. Two types of samples were obtained, namely Endoscopic Ultrasound-Guided Fine-Needle Aspiration (EUS-FNA) biopsies from patients with unresectable tumors, and tumor tissues from patients undergoing surgery. PDAC samples were mixed with 100 μ l of Matrigel (BD Biosciences) and implanted with a trocar (10 Gauge, Innovative Research of America, Sarasota, FL) in the subcutaneous right upper flank of an anesthetized and disinfected mouse. When tumors reached 1 cm^3 , mice were sacrificed and tumors were removed.

To obtain primary cell cultures of these tumors, xenografts were splited into several small pieces and processed in a biosafety chamber: after a fine mincing, they were treated with collagenase type V (ref C9263; Sigma) and trypsin/EDTA (ref 25200-056; Gibco, Life Technologies) and suspended in DMEM supplemented with 1% w/w Penicillin/Streptomycin (Gibco, Life Technologies) and 10% Fetal Bovine Serum (Lonza). After centrifugation, cells were re-suspended in Serum Free Ductal Media (SFDm) adapted from Schreiber et al. [25] and incubated at 37°C in a 5% CO₂ incubator.

Gene expression microarrays

RNAs extraction was performed according to Chirgwin's protocol (Chirgwin et al., 1979). Total RNA (1.0 μ g) was reverse transcribed for hybridization to the human oligonucleotide array Human Gene 2.0 (Genechip, Affymetrix) as described previously [26]. Briefly, arrays were processed using the Affymetrix GeneChip Fluidic

Station 450 (protocol EukGE-WS2v5_450) and scanned using a GeneChip Scanner 3000 G7 (Affymetrix). The GeneChip Operating Software (Affymetrix GCOS v1.4) was used to obtain chip images and for quality control. Background subtraction and normalization of probe set intensities were performed using the method of Robust Multiarray Analysis (RMA) [27]. All array data are available at National Center for Biological Information (NCBI) Gene Expression Omnibus (GEO) omnibus GSE55513.

Chemogram

Cells were screened for their chemosensitivity to 5-aza-dC (ref. A2385 Sigma Aldrich). These cells were treated for 72 h with increasing concentrations of 5-aza-dC ranging from 0 to 80 μ M. Five thousand cells per well were plated in 96-wells plates in SFDM medium. Twenty four hours later the media was supplemented with increasing concentrations of 5-aza-dC and incubated for an additional 72 h period. Each experiment was done in triplicate and repeated at least three times. Cell viability was estimated after addition of PrestoBlue reagent (Life Technologies) for 3 h following the PrestoBlue cell viability reagent protocol provided by the supplier.

RT-qPCR

One μ g RNA from xenografts was reversed transcribed using the Go Script reagent (Promega) according to manufacturer's instructions. Real-time quantitative PCR for DNMT1, DNMT3A and DNMT3B mRNA was performed in a Stratagene cycler using Takara reagents. Primers sequences are available upon request.

In vivo experiments

In vivo experiments were conducted in accordance with institutional guidelines and were approved by the "Plateforme of Stabulation et d'Expérimentation Animale" (PSEA), Scientific Park of Luminy, Marseille. Human-PDAC xenografts were established by subcutaneous implantation of human tumors in the upper right flank of 5- to 6-week-old nude mice (Swiss Nude Mouse Crl: NU(lco)-Foxn1nu, Charles River Laboratories). Matrigel (BD Biosciences) was added to tumors just before implantation and tumors were maintained in mouse by splitting. For the 5-aza-dC (Sigma) treatment the drug was injected at 0.250 mg/kg/daily intraperitoneally (150 μ l per injection) starting at day 21 after implantation. A fragment of 100 mm³ was implanted and the growing of tumors was measured weekly by caliper and calculated as length x width x depth.

Clinical outcome

The 17 patient's clinical outcomes were recorded in a prospective database, and data such as age, gender, medical history, histopathological stage of differentiation,

treatments received, response to chemotherapy, date of progression, metastasis occurrence and localization and death date were analyzed. Progression-free survival and overall survival were then calculated.

Differentiation score

PDAC xenograft score of differentiation was based on the extent of glandular differentiation. If > 95% of the tumor is composed of glands then it is classified as being well differentiated, 50%–95% is moderately differentiated, and < 50% is poorly differentiated or undifferentiated.

Conflict of interest

The authors disclose no conflicts of interest.

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Research Paper

A pancreatic ductal adenocarcinoma subpopulation is sensitive to FK866, an inhibitor of NAMPT

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ABSTRACT

Treating pancreatic cancer is extremely challenging due to multiple factors, including chemoresistance and poor disease prognosis. Chemoresistance can be explained by: the presence of a dense stromal barrier leading to a lower vascularized condition, therefore limiting drug delivery; the huge intra-tumoral heterogeneity; and the status of epithelial-to-mesenchymal transition. These factors are highly variable between patients making it difficult to predict responses to chemotherapy. Nicotinamide phosphoribosyl transferase (NAMPT) is the main enzyme responsible for recycling cytosolic NAD⁺ in hypoxic conditions. FK866 is a noncompetitive specific inhibitor of NAMPT, which has proven anti-tumoral effects, although a clinical advantage has still not been demonstrated. Here, we tested the effect of FK866 on pancreatic cancer-derived primary cell cultures (PCCs), both alone and in combination with three different drugs typically used against this cancer: gemcitabine, 5-Fluorouracil (5FU) and oxaliplatin. The aims of this study were to evaluate the benefit of drug combinations, define groups of sensitivity, and identify a potential biomarker for predicting treatment sensitivity. We performed cell viability tests in the presence of either FK866 alone or in combination with the drugs above-mentioned. We confirmed both inter- and intra-tumoral heterogeneity. Interestingly, only the *in vitro* effect of gemcitabine was influenced by the addition of FK866. We also found that NAMPT mRNA expression levels can predict the sensitivity of cells to FK866. Overall, our results suggest that patients with tumors sensitive to FK866 can be identified using NAMPT mRNA levels as a biomarker and could therefore benefit from a co-treatment of gemcitabine plus FK866.

INTRODUCTION

Treating patients with pancreatic ductal adenocarcinoma (PDAC) is a challenge due to the poor prognosis of this disease and its resistance to chemotherapies. This can be partially attributed to a dense stroma barrier surrounding the tumor and the lack of vessels, which decrease drug delivery to the tumor site, also limiting access to oxygen and nutrients [1]. PDACs also present epithelial-to-mesenchymal transition and the presence of cancer stem cells is a sign of aggressiveness and chemoresistance [2]. Chemoresistance is particularly difficult to overcome due to genetic heterogeneity both between pancreatic tumors, and within each tumor. These factors can render patients completely unresponsive to a given chemotherapy and in those cases that do respond the selection of resistant clones within the tumor can cause cancer relapse. Hypoxic conditions and limited access to nutrients induce cancerous cells to adapt their metabolism to survive and grow in this hostile environment [3]. One of the mechanisms activated is the Warburg's effect, characterized by increased glycolysis and lactate overproduction. This process, is also known as aerobic glycolysis and promotes tumor growth [4]. The high rates of aerobic glycolysis perturb NAD^+ metabolism, altering the NADH/NAD^+ redox ratio. Indeed, instead of eventually transferring electrons from NADH to the mitochondrial respiratory chain, a large proportion of cytosolic NAD^+ is regenerated by reducing pyruvate to lactate. NAD^+ is an essential coenzyme involved in numerous metabolic pathways and energy transduction of nearly every cell. It mediates signaling events and participates in the regulation of many biological processes, including transcription, cell cycle progression, apoptosis, DNA repair and metabolic regulation [5]. Cellular NAD^+ levels have been recognized as a homeostatic process with immediate effects on cellular metabolism. A stable level is maintained by a finely regulated balance between NAD^+ degradation, NAD^+ synthesis and NAD^+ recycled through a salvage pathway. Pancreatic cancer cells rely heavily on the NAD^+ salvage pathway for their metabolism. Two enzymes which play major roles in this pathway are nicotinamide phosphoribosyl transferase (NAMPT) and nicotinamide mononucleotide adenylyl transferase (NMNAT1) [6, 7]. NAMPT is the most active enzyme catalyzing the rate-limiting step of nicotinamide condensation with 5-phosphoribosyl-1-pyrophosphate to yield nicotinamide mononucleotide, then NMNAT1, leading to NAD^+ from nicotinamide mononucleotide and ATP. NAMPT is up-regulated in a number of solid tumors and is involved in angiogenesis by inducing vascular endothelial growth factor [8, 9].

FK866 is a noncompetitive highly specific inhibitor of NAMPT and clinically interesting as it is a potent antitumor drug both *in vitro* and *in vivo* [10]. Many recent studies provide evidence that it selectively

inhibits growth of various kinds of cancer cells, with no effect on normal cells [8]. It causes cellular death by apoptosis [11] and induces autophagy. Its effects on autophagy are potentiated by chloroquine and antagonized by 3-methyladenine or by down-regulating autophagy-related proteins [12, 13]. Interestingly, NAMPT inhibition sensitizes pancreatic adenocarcinoma cells to tumor-selective, PAR-independent metabolic catastrophe and cell death induced by β -lapachone [14] and FK866 led to either tumor regression or stabilization in a preclinical trial [11]. However, in five clinical trials testing three specific NAMPT inhibitors (FK866, CHS828 and GMZ1777), no significant tumor remission was observed in a total of 104 patients. Clinical studies on FK866 have revealed that due to its short half-life in circulation, prolonged treatment regimens are required, inducing toxicity to proliferating hematopoietic cells. Therefore, the efficiency of NAD^+ depleting drugs, such as NAMPT inhibitors, when used alone are expected to be low due to insufficient tumor-selectivity [15-17]. For this reason, FK866 has also been tested as an additive drug to other well-known chemotherapies. It increased the chemosensitivity of gastric cancer cells to 5-Fluorouracil (5FU) [18], potentiated the effects of cisplatin and etoposide in neuroblastoma cell lines [13] and massively reduced the overall metabolic activity in xenografts, impairing PDAC growth [19].

Combining drugs to treat chemoresistant cancers, such as PDAC, subsequently became a promising alternative. The best example in PDAC was the advent of Folfirinox (5FU, oxaliplatin and irinotecan) that produced the longest improvement in survival ever seen in a phase III clinical trial of patients with advanced pancreatic cancer [20, 21]. This drug combination led to increased cell death, a reduction of drug resistance, while allowing the use of lower doses and therefore fewer side effects. Here, we sought to increase the efficiency of killing pancreatic cancer cells via an association of drugs which target two different cellular processes (i.e.: metabolism and genomic stability). We investigated the effect of the NAMPT inhibitor, FK866, on PDAC-derived primary cell cultures (PCCs) and determined whether it can potentiate the effects of three frequently used chemotherapeutic agents: gemcitabine, 5FU and oxaliplatin. We also attempted to determine groups of sensitivity to these drug combinations and identify a screening biomarker (companion test) capable of predicting this sensitivity.

RESULTS

FK866 sensitivity is heterogeneous in PCCs from PDAC patients

Twenty-three PDAC patients were included in this study and patient's distribution representing a normal PDAC cohort is indicated in Table 1. An increase in the

Table 1: Patients Distribution: Clinical characteristics for the 23 patients included in the study

Patients Distribution		
Patients		23
Sex	Male	16 (70%)
	Female	7 (30%)
Age	Mean	65
	Median (Min-Max)	66 (42-87)
Personal History of Cancer	No	18 (79%)
	Yes	5 (21%)
Surgery		12 (52%)
Unresectable		11 (48%)
Tumor Location	Head	14 (61%)
	Neck	1 (4%)
	Body	0
	Tail	8 (35%)
Locally Advanced		3 (13%)
Metastasis	Liver	4 (17%)
	Lung	2 (8.5%)
Tumor Status at Diagnosis	Carcinomatosis	2 (8.5%)
	Stage I	0
	Stage IIA	2 (8.5%)
	Stage IIB	9 (40%)
	Stage III	4 (17%)
	Stage IV	8 (34.5%)
Overall Survival (Months)	Median (Min-Max)	12.1 (1.2-38.8)

number of operated patients was observed due to the fact that xenografts from surgical specimens grow better than biopsies. PCCs were obtained from patient-derived xenografts in nude mice. These cells were submitted to increasing concentrations of FK866 (from 0 to 1000 nM) to determine their sensitivity by plotting dose-response curves. We established a chemogram (i.e.: determination of the cell viability as a function of the chemotherapeutic drug concentration) for each patient-derived PCC. Using this approach we were able to estimate their relative chemosensitivity by comparing their half maximal inhibitory concentrations (IC50s). Notably, the three PCCs most sensitive to FK866 were: HN-01 (IC50 = 0.41 nM), 01.030 (IC50 = 0.30 nM) and C-NOR (IC50 = 0.85 nM), whereas 02.058, HN-03 and AD-IPC were the three most resistant, all with IC50s > 1000 nM (Figure 1a). This manner of analyzing data could not determine scores for those PCCs that did not achieve an IC50, even at very high

FK866 concentrations. We observed maximal variability in the sensitivity of PCCs to FK866 at a concentration of 3.9 nM. We next decided to score the PCCs sensitivity based on cell viability following treatment with 3.9 nM FK866 (Figure 1b). HN-01, 01.030 and C-NOR remained the most sensitive PCCs, however the most resistant were: L-NOR, AR-IPC and E-NOR. We used this scoring method for the next experiments in this study. Overall, our data reveals that each PDAC-derived PCC has its own sensitivity to FK866 with a huge range of IC50s (from 0.30 to >1000 nM), suggesting a very high response variability among patients. Importantly, the dose-response curves depict a cell viability plateau for almost every PCC, even with high doses of FK866, indicating intra-tumoral heterogeneity in terms of sensitivity to the FK866 treatment (Figure 1). As sensitivity to FK866 could be associated with PCC replication time, this was calculated and analyzed. Doubling times varied between 16.46±5.33h

for L-IPC and 44.63 ± 6.07 h for 02.087p (Supplementary Figure 1). All PCCs actively replicate during the time of chemosensitivity determination and no correlation was observed between doubling time and FK866 sensitivity and between doubling time and NAMPT expression level (data not shown). We also analyzed clinical characteristics of FK866 sensitive and resistant patients and we did not find any correlation.

FK866 increases sensitivity to gemcitabine, but not 5FU or oxaliplatin, in a group of PCCs from PDAC patients

In order to study FK866 in combination with other drugs, we tested two different FK866 concentrations (0.06 and 3.9 nM) with a series of 11 increasing concentrations of three genotoxic drugs (5FU, oxaliplatin and gemcitabine). We selected a concentration of 0.06 nM FK866 as this was the maximal dose that caused a minimal effect in all cells tested. Conversely, the dose of 3.9 nM caused the most variable effect between the 23 PCCs (see Figure 1). We decided to continue the study

using the 4 most sensitive PCCs to FK866 (01.030, C-NOR, L-IPC and HN-01). A combination of 0.06 nM FK866 with different gemcitabine concentrations had no significant effect on cell viability (Figure 2a). However, 3.9 nM FK866 significantly decreased cell viability in all 4 sensitive PCCs tested. We therefore concluded that treatment with 3.9 nM FK866 can enhance the effect of gemcitabine, which was not observed at the lower concentration (0.06 nM). The PrestoBlue method we used to measure cell viability is based on mitochondrial activity (see Material and Methods). As FK866 could influence mitochondrial activity, potentially inducing misinterpretation of data, we decided to compare results obtained with PrestoBlue™ with another method that measures living cells by their protease activity (CellTiter-Fluor™). As shown in Figure 2b, the 6 PCCs tested (HN-03, J-IPC, 01.030, C-NOR, Foie 8B and AD-IPC) using two concentrations of FK866 (1 and 3.9 nM), confirmed that results obtained with PrestoBlue™ and CellTiter-Fluor™ are not significantly different. Therefore all measurements of cell viability were subsequently carried out using the PrestoBlue™ method. We next analyzed

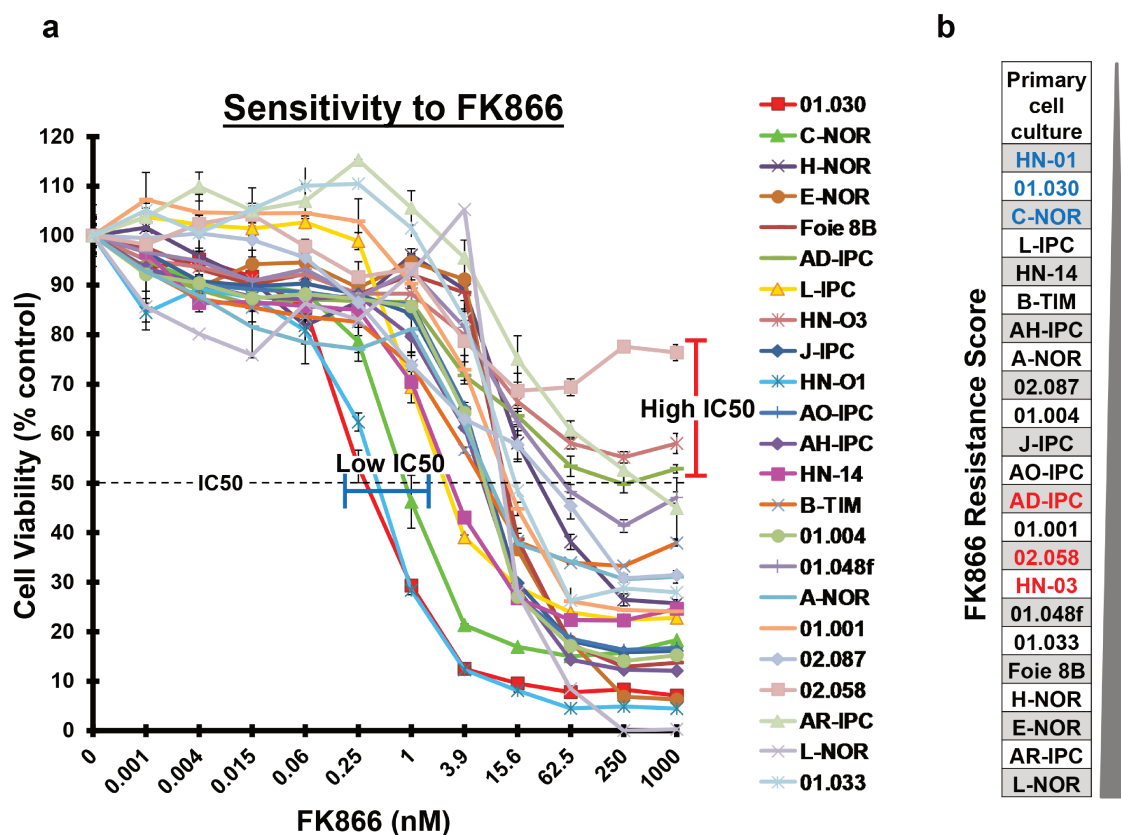


Figure 1: FK866 sensitivity of 23 PDAC-derived PCCs. a. Twenty-three PDAC-derived PCCs were treated for 72 h with increasing concentrations of FK866 ranging from 0 to 1000 nM. The horizontal dotted line indicates 50% cell viability. PCCs with the highest sensitivity are highlighted by a blue line and those with an $IC_{50} > 1000$ nM by a red line. **b.** PCCs were scored on their resistance to a fixed FK866 concentration of 3.9 nM, from the most sensitive to the most resistant. PCCs in blue are the ones that present the lowest IC_{50} and those in red have an $IC_{50} > 1000$ nM.

the effect of combining FK866 with gemcitabine on 7 PCCs that show different degrees of resistance to FK866 (01.001, 01.033, J-IPC, Foie 8B, AR-IPC, 02.058 and 02.087) and observed an important heterogeneity (Figure 3). For PCCs 01.001, 01.033 and J-IPC, there was a clear synergistic effect of combining the drugs, which was not apparent for the PCCs AR-IPC, 02.058, 02.087 and Foie 8B. Most of the PCCs tested (8 out of 11 PCCs) were more sensitive to this co-treatment.

In another set of experiments, 5FU and oxaliplatin were both combined with FK866 at either 0.06 or 3.9 nM. Figure 4 shows results obtained using the PCCs Foie 8B, 01.030, J-IPC and HN-03. Curiously, a combination of NAMPT inhibitor with either 5FU or oxaliplatin resulted in no significant effect, with the exception of 01.030, although only at high concentrations of oxaliplatin (15.6 and 62.5 nM). Interestingly, neither 5FU nor oxaliplatin are clinically used as a monotherapy since they have proven to be almost non-effective, contrary to gemcitabine. Therefore, addition of FK866 could be more

advantageous in combination with gemcitabine treatment instead of 5FU or oxaliplatin.

NAMPT transcriptional level predicts FK866 sensitivity

Next, we hypothesized that NAMPT, as a specific target of FK866, could predict drug sensitivity. We therefore quantified NAMPT at the transcriptional level in PCCs by quantitative real-time PCR (RT-qPCR). We plotted the mRNA expression level in arbitrary units from 0 to 15 (Figure 5a) and used IC₅₀ values to reflect and compare the global sensitivity of PCCs. As shown in Figure 5a, all PCCs expressing over 2 arbitrary units of NAMPT presented IC₅₀s > 3.9 nM. On the contrary, only 53% of PCCs (8 out of 15) showed IC₅₀s > 3.9 nM with an NAMPT expression level of less than 2 arbitrary units. Therefore, our results indicate that resistance to FK866 positively correlates with the expression level of NAMPT transcript. In Figure 5b we plotted results obtained by a

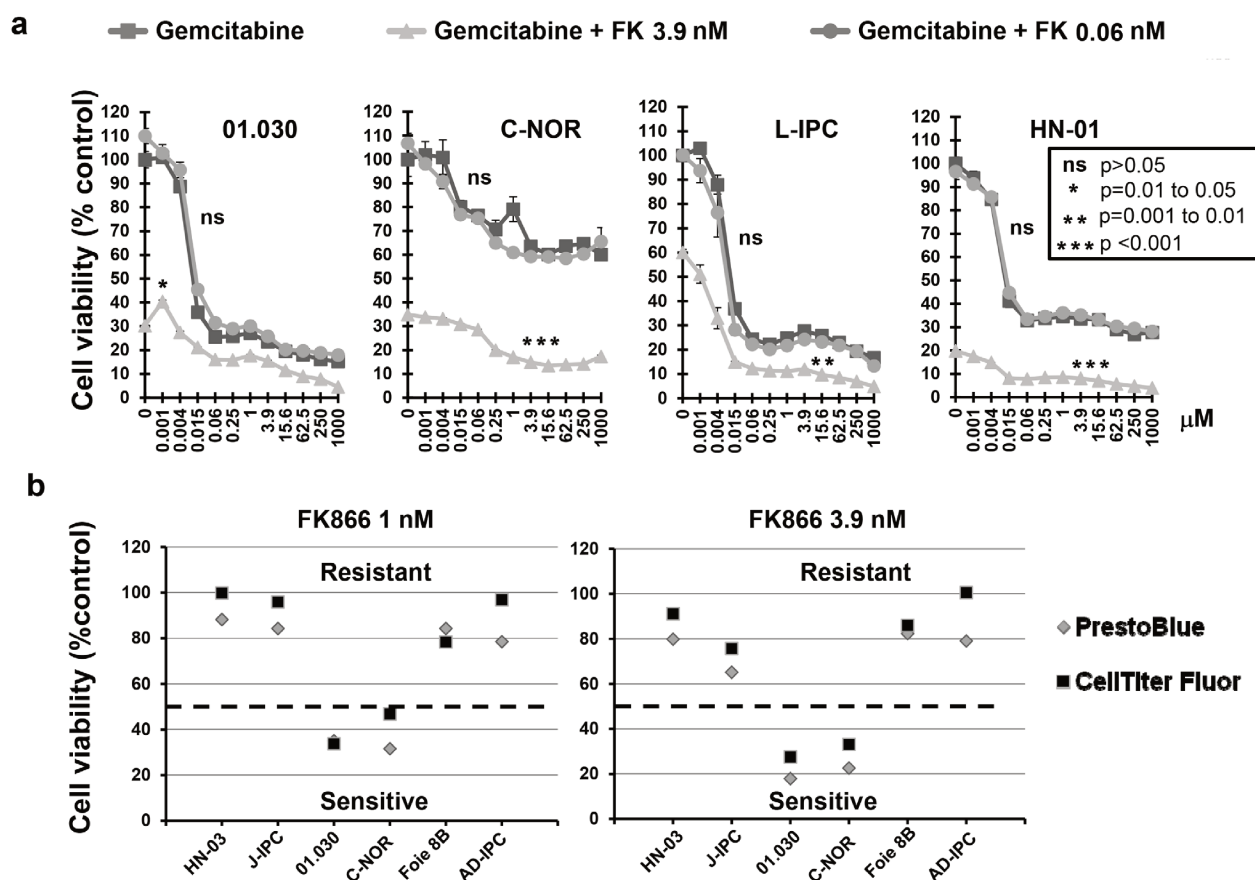


Figure 2: a. Effect of FK866-gemcitabine co-treatment on four PCCs most sensitive to FK866. Dose response curves represent cell viability with increasing concentrations of either gemcitabine alone or in combination with two concentrations of FK866 (0.06 and 3.9 nM). Three independent experiments were done (each one in triplicate) showing similar results. Significant difference between curves ($p \leq 0.05$) was analyzed by the Mann-whitne test. **b.** Comparison of cell viability measured with either PrestoBlue™ or Cell Titer-Fluor™ after FK866 treatment. Cell viability was measured with either PrestoBlue™ or Cell Titer-Fluor™ after 3 days treatment with two concentrations of FK866 (1 and 3.9 nM). The dotted horizontal line indicates 50% cell viability.

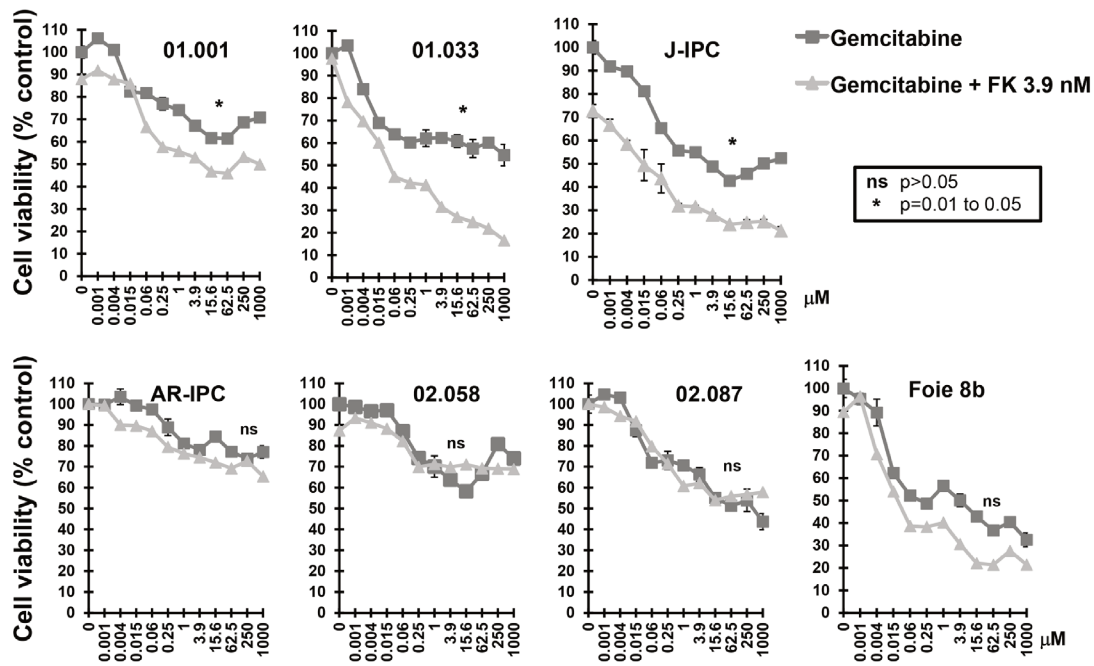


Figure 3: Sensitivity of FK866 resistant PCCs to co treatment with FK866-gemcitabine. Dose response curves represent cell viability with increasing concentrations of gemcitabine either alone or in combination with two concentrations of FK866 (3.9 nM). Three independent experiments were done (each one in triplicate) showing similar results. Significant differences between curves ($p \leq 0.05$) were analyzed by the Mann-whitne test.

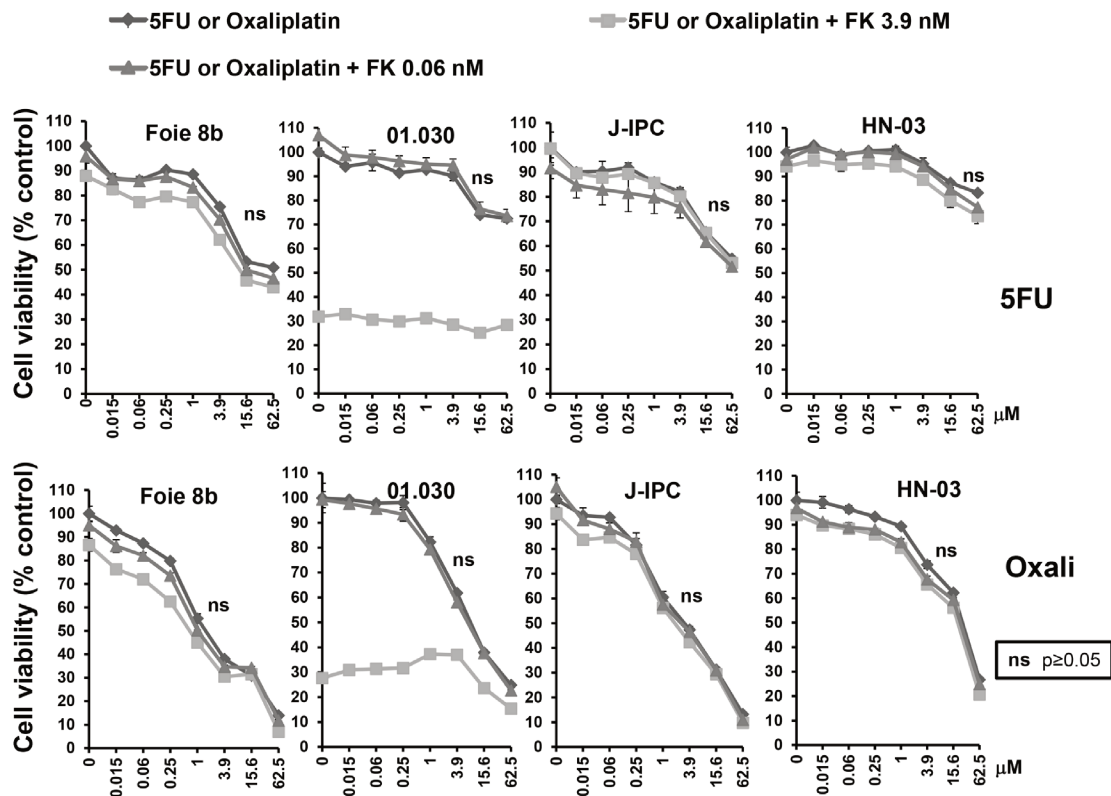


Figure 4: Sensitivity of PCCs to co-treatment with FK866-5FU and FK866-oxaliplatin. Dose response curves represent cell viability at increasing concentrations of 5FU or oxaliplatin, either alone or in combination with two concentrations of FK866 (0.06 and 3.9 nM). Three independent experiments were done (each one in triplicate) showing similar results. Significant differences between curves ($p \leq 0.05$) were analyzed by the Mann-whitne test.

regression analysis between NAMPT mRNA expression and FK866 IC50 in PCCs. We observed a positive correlation, indicating that PCCs which express higher levels of NAMPT have an increased resistance to FK866 treatment. These results strongly suggest that the primary culture of pancreatic epithelial cells does not significantly modify NAMPT transcriptional expression. In some cases we observed PCCs (especially E-NOR or AD-IPC) with low NAMPT mRNA expression and high resistance to FK866, we have no clear explanation for that but we observed that in all cases highly NAMPT expressing cells have $IC_{50} > 3.9$. We also analyzed the putative association between patient survival Vs NAMPT expression and Vs FK866 sensitivity and we did not find any correlation. We analyzed other clinical characteristics in particular

differentiation status of xenograft and we did not find any association.

Metabolic status of PDAC-derived cells does not correlate with NAMPT expression level

NAD^+ is a coenzyme which plays an essential role in metabolism within tumoral cells. Its synthesis is accomplished by a salvage pathway implicating NAMPT. Since NAMPT expression level and sensitivity to FK866 correlate, we hypothesized that tumoral cells with a high metabolism, i.e consuming or producing more metabolites with higher levels of NAMPT, would die at a faster rate than metabolically neutral cells. We therefore analyzed the relationships between either NAMPT expression or FK866 sensitivity with glucose/glutamine

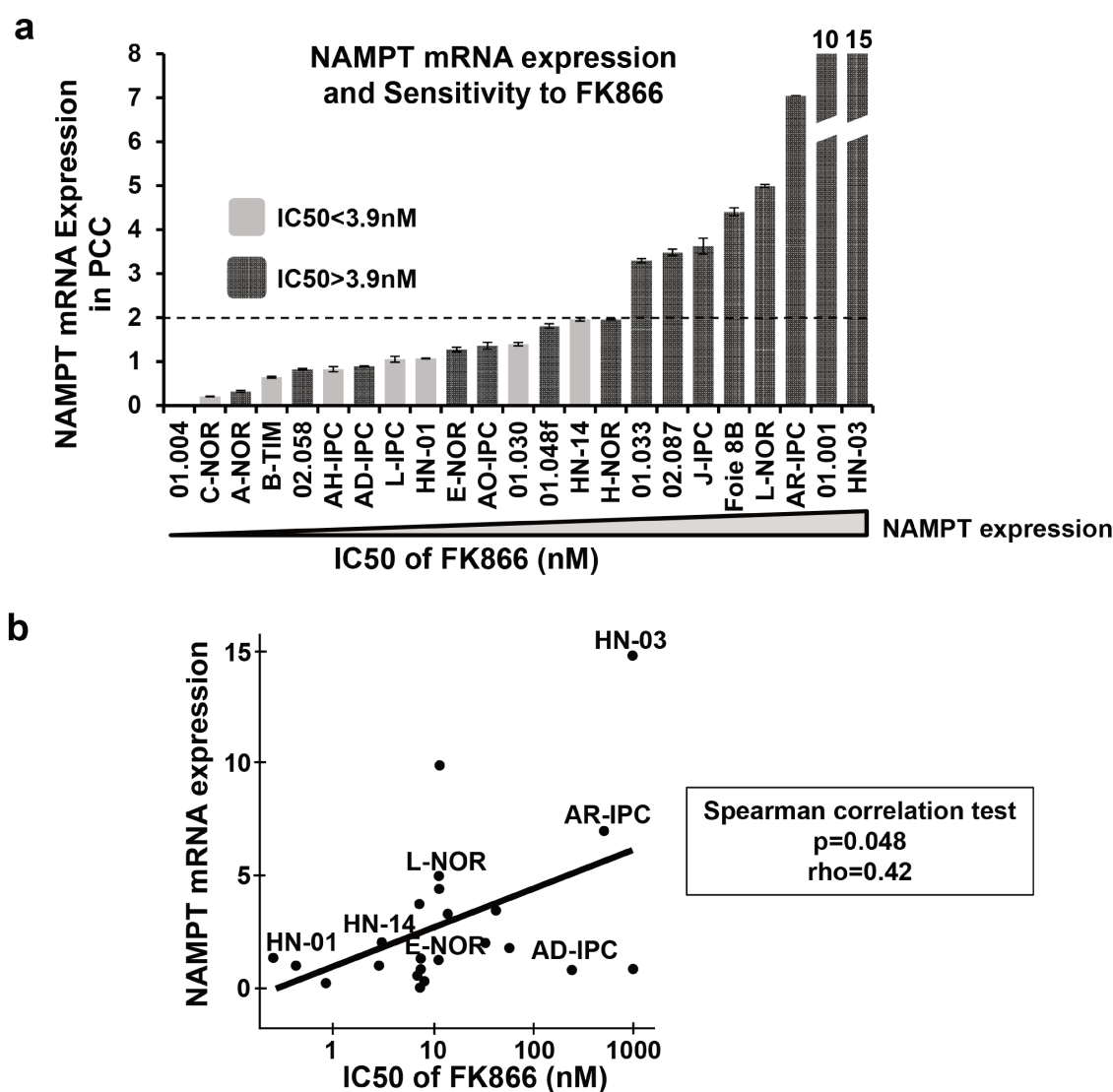


Figure 5: NAMPT mRNA expression levels in PCCs. a. PCCs were ordered according to their NAMPT expression level as determined by RT-qPCR (the lowest NAMPT expression level is on the left; highest on the right). The dark grey bars are FK866 resistant PCCs ($FK866 IC_{50} > 3.9 \text{ nM}$); light grey bars are FK866 sensitive PCCs ($FK866 IC_{50} < 3.9 \text{ nM}$). **b.** Correlation between FK866 IC_{50} and NAMPT mRNA expression for the 23 PCCs with Spearman correlation test ($p=0.048$); $\rho=0.42$.

consumption and lactate/glutamate production for the 23 PCCs (Figure 6). We found no correlation between these metabolic parameters and FK866 sensitivity. Contrary to our hypothesis, high metabolism does not predict either FK866 sensitivity or NAMPT expression.

Higher sensitivity of PDAC-derived cells to co-treatment (gemcitabine + FK866) compared to gemcitabine alone

We next studied the sensitivity of PCCs to treatments with gemcitabine alone versus gemcitabine plus FK866 in all 23 PCCs. Gemcitabine was used at a concentration of 62.5 μ M, demonstrated to be efficient in the chemograms, and FK866 at 3.9 nM (Figure 7a). As shown in Figure 7b, the combined treatment (gemcitabine + FK866) synergistically decreased the cell viability of 16 PCCs from 23 (69.6%), each with a ratio of > 1 (cell viability after treatment with gemcitabine alone

/ cell viability after combined gemcitabine + FK866). However, we observed no correlation between this benefit of combined treatment and NAMPT expression levels. We then analyzed the effect of FK866 alone or combined with gemcitabine on the intracellular levels of NAD^+ (see Material and Methods). As shown in Figure 7C, 02.058 and AR-IPC cells which are resistant to FK866 and do not show any decrease in their viability after gemcitabine + FK866 treatment present high NAD^+ levels in all conditions (treated or not with FK866 or with FK866 + gemcitabine). Differently, cells resistant to FK866 and with a decrease in their viability after combined treatment FK866+gemcitabine (J-IPC, 01.033) as well as FK866 sensitive cells (HN-01 and C-NOR) show low levels of NAD^+ in all conditions. We finally examined clinical characteristics of patients that could take advantage of a combined treatment against the others and we did not find any common characteristic between them. Overall, these results suggest that most PDAC patients could take

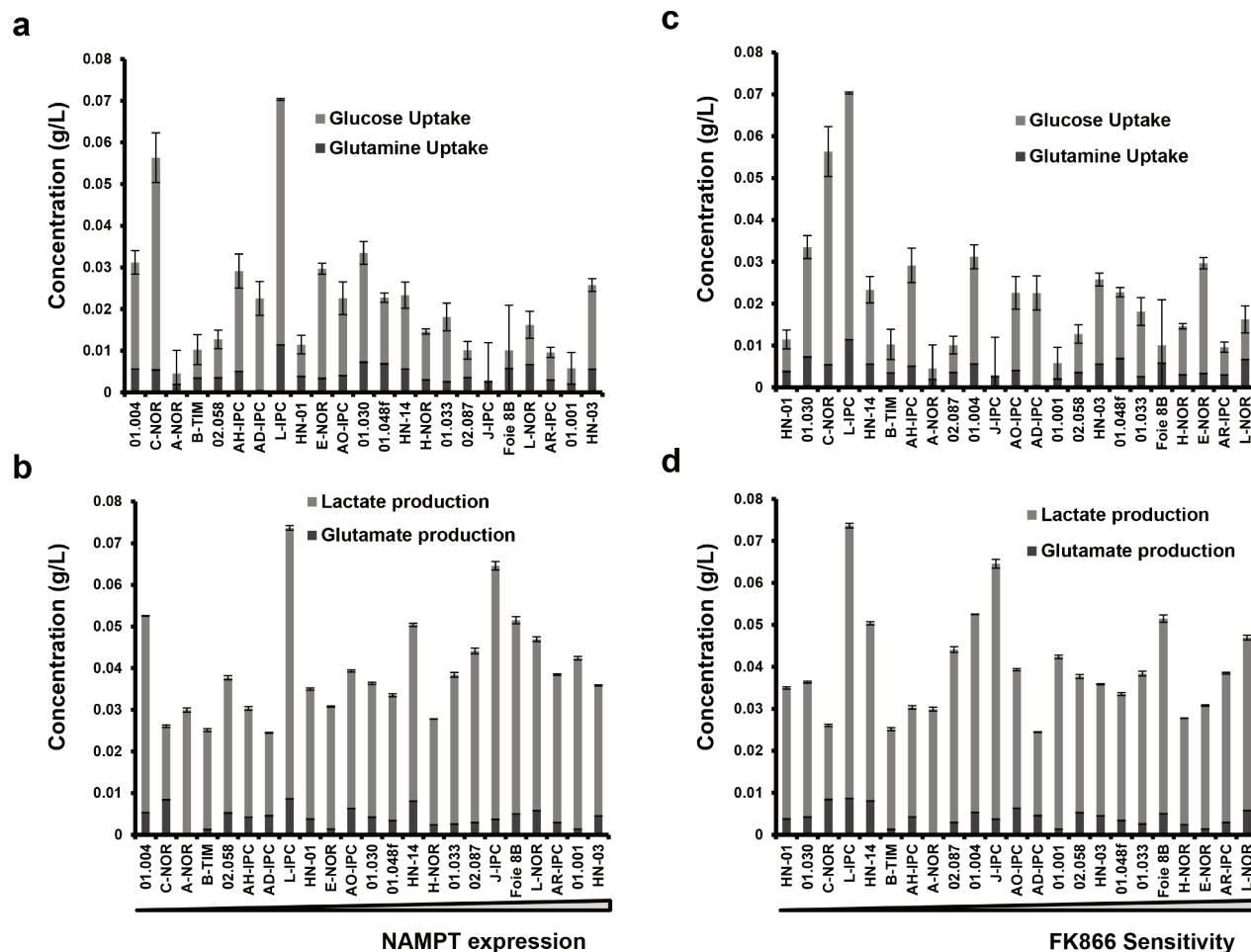


Figure 6: Correlation between NAMPT expression or FK866 sensitivity with either glucose/glutamine uptake or lactate/glutamate production. Primary cell cultures were ordered according to either their NAMPT transcriptional level **a** and **b**, or their FK866 IC50 **c** and **d**. For each of them we measured glucose and glutamine accumulated uptake, as well as lactate and glutamate production. The values obtained were normalized by the amount of protein.

advantage of co-treatment with gemcitabine + FK866. In addition, quantification of NAMPT mRNA expression could be used as a potential biomarker for determining the sensitivity of tumors to FK866, despite NAMPT levels not predicting a benefit of a combined treatment of gemcitabine-FK866. Interestingly high NAD⁺ levels seem to designate cells that will not present a decrease in cell viability with the FK866-gemcitabine combined treatment.

DISCUSSION

The main aims of this study were to define whether there are differences in sensitivity of PDACs to a metabolic drug either alone or in combination; and if so, to determine a way to identify the most sensitive

patients by characterizing tumors at the molecular level. The results presented in this paper have revealed a group of tumors that appear more sensitive to FK866, a specific inhibitor of NAMPT. An interesting aspect of this work is the efficient strategy we employed to include 23 consecutive patients with PDAC tumors in the study by collecting samples from both surgical specimens and EUS-FNA biopsies and preserving them as xenografts in immunosuppressed mice. We studied NAMPT mRNA expression profiles of these patient-derived PCCs using RT-qPCR and found a correlation between NAMPT expression and sensitivity to FK866. Finally, and most importantly, we discovered that FK866 improves the effect of gemcitabine *in vitro*, although not 5FU or oxaliplatin.

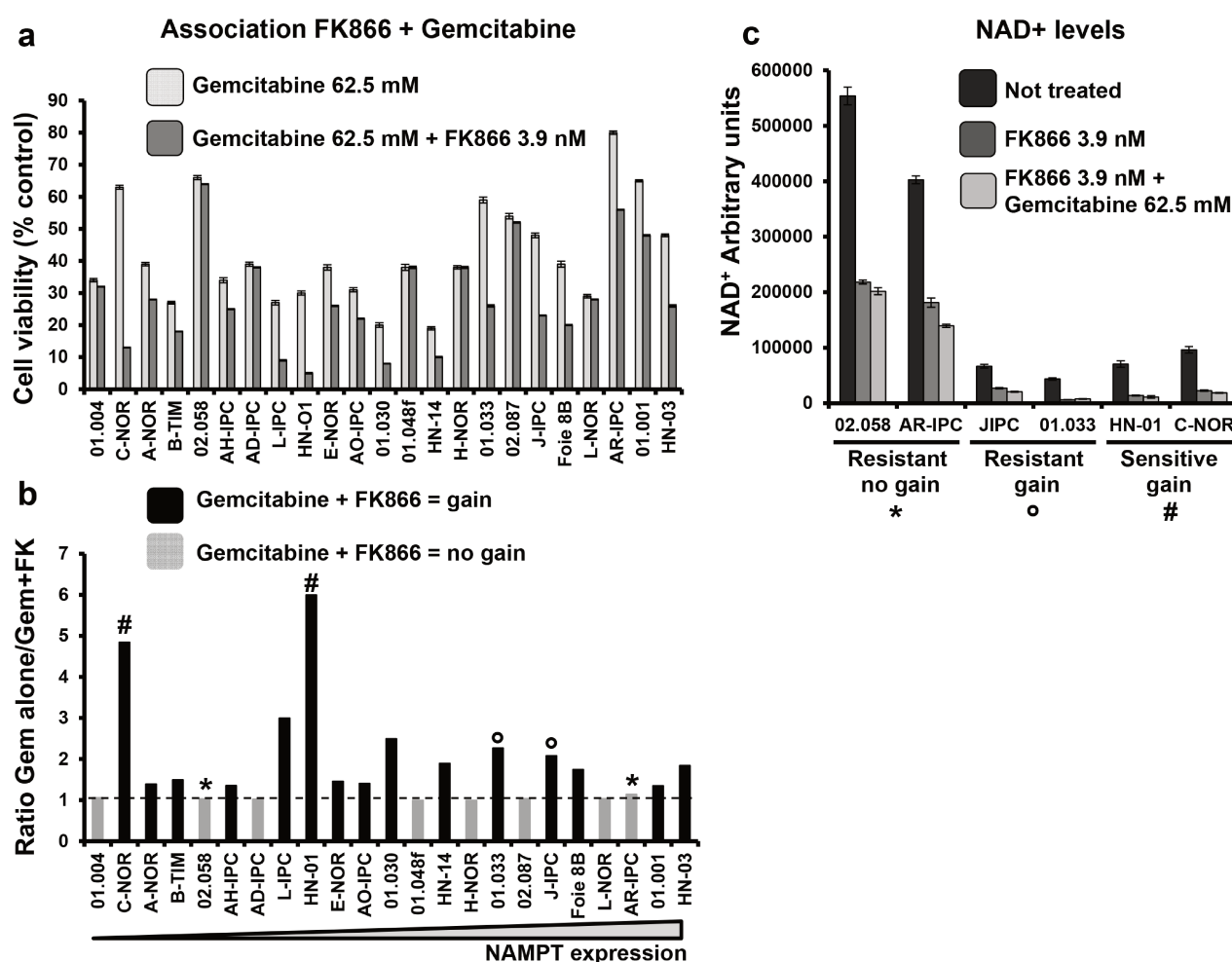


Figure 7: Co-treatment of FK866-gemcitabine. **a.** Cell viability was measured and indicated as percentage of the control (untreated) after 3 days treatment with either 62.5 mM gemcitabine or 62.5 mM gemcitabine + 3.9 nM FK866. **b.** Ratio of treatment with gemcitabine alone / gemcitabine + FK866. The transversal line indicates ratio = 1. Values > 1 indicate a gain with the combined treatment and are represented by black bars on the graph. No gain from co-treatment is indicated by the light grey bars. **c.** NAD⁺ levels measured in: FK866 resistant cells presenting gain (*) or not (o) after treatment with gemcitabine; and FK866 sensitive cells (#). Cells were treated or not with FK866 alone or with FK866 + gemcitabine and NAD⁺ levels measured 24h later. Values are presented as arbitrary units and were normalized to cell number (see Material and Methods). Experiments were repeated 3 times and performed in triplicate.

NAMPT is a fascinating target for the chemotherapeutic treatment of PDAC for a number of reasons. Firstly, the recycling of NAD⁺ is vital for cancer cells due to a high need of ADP ribosylation to repair DNA, preserve genome stability, and maintain telomeres [22]. This is why NAD⁺ dependence renders cancer cells more sensitive to NAMPT inhibition than normal cells. Secondly, NAMPT allows NAD⁺ synthesis by a salvage pathway in a dose dependent manner, and NAMPT is known to be increased in several cancers [23]. Finally, NAD⁺ synthesis is increased by DNA damage in primary culture cells [24].

FK866 is an interesting compound since its mechanism of action is well established and several clinical trials have been performed in patients with different cancers, including several solid tumors. Previous studies indicate that metabolic reprogramming is not only the consequence of cancer cells proliferation and growth; it is a direct function of oncogenesis and consequently an oncogenesis motor [25, 26]. Cancer cells are also NAD-dependent as NAD⁺ is an important cofactor in the oxidative phosphorylation chain as well as a substrate for enzymes involved in genomic stability, apoptosis, cell signaling, stress tolerance and metabolism. NAMPT is the main enzyme responsible for maintaining NAD⁺ levels in tumors and may be the “Achilles’ heel” to study for future chemotherapy.

An important point to note from our study is that the IC₅₀ for FK866 in patient-derived PCCs varied greatly from 0.295 nM to >1000 nM. A group of patients expressing high levels of NAMPT mRNA were relatively resistant to the treatment with FK866 suggesting that its expression was not completely inhibited by FK866, perhaps for a stoichiometric reason. Accordingly, we found that PCCs expressing intermediary or lower levels of NAMPT mRNA were frequently associated with higher levels of sensitivity to the treatment with FK866. We hypothesize that this group with higher sensitivity is dependent on NAMPT levels and the amount expressed by the cell can be inhibited by FK866. Altogether, our data suggest that levels of NAMPT in human tumors can be used to predict sensitivity to FK866 treatment. Consistent with this, it has been previously suggested that NAMPT expression could play a role in the response to FK866 [5, 19]. For example, on fibrosarcoma cells NAMPT overexpression has been shown to protect cells against FK866 treatment [27]. Furthermore, NAMPT inhibition using a siRNA in the presence of GMX1778, another NAMPT inhibitor, increased apoptosis of different cancer cells. In addition, NAMPT expression showed an inverse correlation to GMX1778 toxicity [28].

It is unlikely that NAMPT inhibition would be solely used as a therapeutic approach for treating patients with a PDAC since FK866 at high concentrations is very toxic, as anticipated from its mechanism of action which affects basic functions of both cancerous and normal

cells. Therefore, the only possibility is to use FK866 in combination with cytotoxic drugs to potentiate their effect. Accordingly, the second point to be noted from this work is the significant potentiation of gemcitabine on cell viability by co-treatment with FK866, yet surprisingly, this added benefit was almost negligible when combined with 5FU or oxaliplatin. This was unexpected since previous studies have shown a potentiation of the effects of 5FU by FK866 on gastric cancer cells and of cisplatin by FK866 on neuroblastoma cells [13, 18]. These results suggest that the enhancement of the effects of cytotoxic drugs by FK866 is most likely dependent on cancer cell type. Moreover intracellular NAD⁺ depletion after cell treatment with FK866 induces autophagy [12, 29]. VMP1-Mediated Autophagy is also induced by gemcitabine promoting apoptosis [30]. Both effects could be additive increasing cell death and explaining the synergistic effect of FK866 and gemcitabine. Another putative explanation could be that gemcitabine induces the DNA damage response and in consequence PARP-1 activity [31]. PARP-1 needs NAD⁺ as a substrate for auto poly-ADP-ribosylation (PARylation) and activation. PARPs are highly responsive proteins; their enzymatic activity can increase up to 500-fold after DNA consuming up to 80–90% of the intracellular NAD⁺ stores [32]. As NAD⁺ is depleted by FK866 treatment, PARP-1 could become inactive and thus increase the effect of gemcitabine on pancreatic cancer cells. On another hand differently to gemcitabine, autophagy induction protects cancer cells against oxaliplatin and 5FU-induced cell death [33, 34]. This could explain the absence of synergistic effect combining Oxaliplatin and 5FU with FK866 on pancreatic cancer primary cultures.

FK866 has been shown to exert strong effects on cancer cell metabolism, such as glycolysis, pentose phosphate pathway, tricarboxylic acid cycle, serine biosynthesis and attenuating glycolysis at the glyceraldehyde 3-phosphate dehydrogenase step due to reduced availability of NAD⁺ for the enzyme [35]. We therefore hypothesized that FK866 could be more efficient in tumors with increased glycolysis or glutaminolysis. However, contrary to this we were unable to demonstrate a correlation between either glucose/glutamine consumption or lactate/glutamate production and sensitivity levels of PCCs to FK866 treatment. This suggests that pancreatic cancer cell metabolism is not mandatory to the sensitivity to FK866 treatment. Nevertheless some particular points are worth discussing, in particular the one observed for the L-IPC tumor that presents the highest lactate production. This patient was diagnosed with an anaplastic carcinoma of the pancreas, one of the most aggressive forms of pancreatic cancer and his survival time after diagnosis was of only 5.28 month. On the other hand J-IPC presents one of the highest survival times in this cohort (25.74 months). These two cases represent interesting but only isolated observations and more of these uncommon patients would be necessary to include in the study in order to draw solid

conclusions about pancreatic cancer aggressiveness and metabolism.

Finally, we tested the efficiency of NAMPT in depleting NAD⁺ (Figure 7c). Cells presenting higher basal levels of NAD⁺, also presented elevated levels of NAD⁺ after FK866 and FK866 + gemcitabine treatments. Interestingly FK866 sensitive and resistant cells showing a decreased viability after gemcitabine treatment also showed lower levels of NAD⁺ before and after the different treatments, suggesting that basal levels of NAD⁺ in pancreatic tumoral cells could predict if the patient will take advantage of a combined FK866 and gemcitabine treatment. Tumoral cells with high NAD⁺ basal levels should be resistant to FK866 and did not show any decrease in viability with a combination FK866 + gemcitabine in contrast to FK866 alone.

In addition, our results present a clinical interest since RT-qPCR for measuring NAMPT mRNA expression can be routinely performed either on systematic echo-endoscopy-guided biopsies at diagnosis or on specimens after surgery. A simple and reproducible test to predict the sensitivity of a tumor to FK866 would greatly aid clinicians in their daily decisions of treatment. With lower doses of combined drugs, patients would be partly spared from side effects while receiving an appropriate and personalized treatment. Patients needing gemcitabine to treat a metastatic or an operated PDAC could therefore benefit from a co-treatment of FK866-gemcitabine.

In conclusion, our data demonstrate that: the IC₅₀ of FK866 to kill PCCs varied more than 4000 fold between sensitive and resistant cells; sensitivity to FK866 is dependent on an optimal cellular level of NAMPT mRNA expression which could be exploited as a companion test to select suitable patients for treatment; and finally, FK866 potentiates the effect of gemcitabine on PDAC-derived PCCs.

MATERIALS AND METHODS

PDAC samples and cell culture

Consent forms of informed patients were collected and registered in a central database. The tumor tissues used for xenograft development were deemed excess to that required for the patient's diagnosis. Two types of samples were obtained: endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) biopsies from patients with unresectable tumors; and tumor tissues from patients undergoing surgery. A total of 23 patients were included, all without treatment before biopsy or surgery. PDAC samples were mixed with 100 µl of matrigel (BD Biosciences) and implanted with a trocar (10 Gauge, Innovative Research of America, Sarasota, FL) into the subcutaneous right upper flank of an anesthetized and disinfected mouse. When tumors reached 1 cm³, mice were sacrificed and tumors removed.

To obtain primary cell cultures of these tumors, xenografts were split into several small pieces and processed in a biosafety chamber: after a fine mincing, they were treated with collagenase type V (ref C9263; Sigma) and trypsin/EDTA (ref 25200-056; Gibco, Life Technologies) and suspended in DMEM supplemented with 1% w/w penicillin/streptomycin (Gibco, Life Technologies) and 10% fetal bovine serum (Lonza). After centrifugation, cells were re-suspended in serum free ductal media (SFDM) adapted from Schreiber *et al.* [36] without antibiotic and incubated at 37°C in a 5% CO₂ incubator. Primary cultured cells were splitted many times until obtaining 4x10⁷ cells (representing 20 vials with 2x10⁶ cells each). This was considered passage zero. At this stage only human cancerous epithelial cells were present in the culture. Cells were then splitted a maximum of 6 more times to avoid other alterations associated with passages. Mouse contaminant cells do not replicate any more after a few passages.

Chemograms

Cells were screened for their chemosensitivity to FK866 (Sigma-Aldrich, France). They were grown in SFDM without nicotinamide for 72 h. Five thousand cells per well were plated in 96-well plates in SFDM medium without nicotinamide. Twenty-four hours later the media was supplemented with increasing concentrations of FK866 and incubated for an additional 72 h period. Each experiment was done in triplicate and repeated at least two times. These cells were treated for 72 h with increasing concentrations of FK866 ranging from 0 to 1000 nM (in a progression by a factor 4). FK866 was next tested at a given concentration in association with increasing concentrations of gemcitabine, oxaliplatin or 5FU (0 to 1000 µM).

Viability assays

Cell viability was estimated after addition of PrestoBlue™ reagent (Life Technologies) for 3 h, following the PrestoBlue™ cell viability reagent protocol provided by the supplier. Live-cell protease activity was also measured using CellTiter-fluor™ (Promega, France). For both techniques cell viability was measured by fluorescence. FK866 sensitivity was close for each primary cell culture if cell viability was estimated with either PrestoBlue™ or CellTiter-Fluor™. PrestoBlue™ monitors mitochondrial function alteration as an indicator of cellular necrosis and apoptosis. Cells with an active metabolism reduce resazurin to resorufin due to NADPH, FADH and NADH electrons. It causes a color change detected by fluorometry. Following the strategy published in 2013, cell viability was measured using CellTiter-Fluor™ as an alternative and unrelated method [37]. Since

similar results were observed from the two methods, data was obtained in this paper using PrestoBlue™.

Metabolite consumption and production

Glucose and glutamine uptake and lactate production by cells were quantified in the cell medium after 48 h of incubation. Glutamate production was quantified in cell lysate 48 h after medium change. Measurements were carried out with the enzymatic membrane system bioanalyser robot YSI 2950 (Biochemistry Analyser, Life Sciences). Results were normalized to protein amount.

NAD⁺ measurement

NAD⁺ was measured using the NAD⁺/NADH™ bioluminescent Assay (Promega). Cells were chosen according to their response to FK866 and FK866+Gemcitabine treatments. 3 groups: 1-cells resistant to FK866 that do not present any differences in viability when treated additionally with gemcitabine (02.058; AR-IPC); 2-Cells that are resistant to FK866 and that present a decrease in viability when treated with gemcitabine (J-IPC; 01.033) and 3-cells that are sensitive to FK866 and that show a decreased viability when treated with gemcitabine (HN-01; C-NOR) see Figures 2 and 3. Five thousand cells were seeded in each well of 96 well-plates, culture medium used was Nicotinamide-free SFDM. Twenty-four hours after plating, culture media was supplemented with 3.9nM FK866 or a combination of 3.9nM FK866 and 62.5μM Gemcitabine. NAD⁺ was measured twenty-four hours later. NAD⁺ values were normalized according to viable cell number after treatment.

RT-qPCR

One μg RNA from xenografts was reverse transcribed using the Go Script reagent (Promega) according to manufacturer's instructions. RT-qPCR for NAMPT mRNA was performed in a Stratagene cyler using Takara reagents. Primer sequences were specific to the gene NAMPT (forward 5'-TTCGGTTCTGGTGGAGGTTT-3', reverse 5'-AGACGTTAATCCCAAGGCCA-3').

Statistical analysis

Data are expressed as means ± SD from at least two independent experiments. Data were analyzed using an unpaired t test. Significance was set at p<0.05.

The manuscript has been revised for the English by an independent scientific language editing service.

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CONFLICTS OF INTEREST

The authors disclose no conflicts of interest.

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